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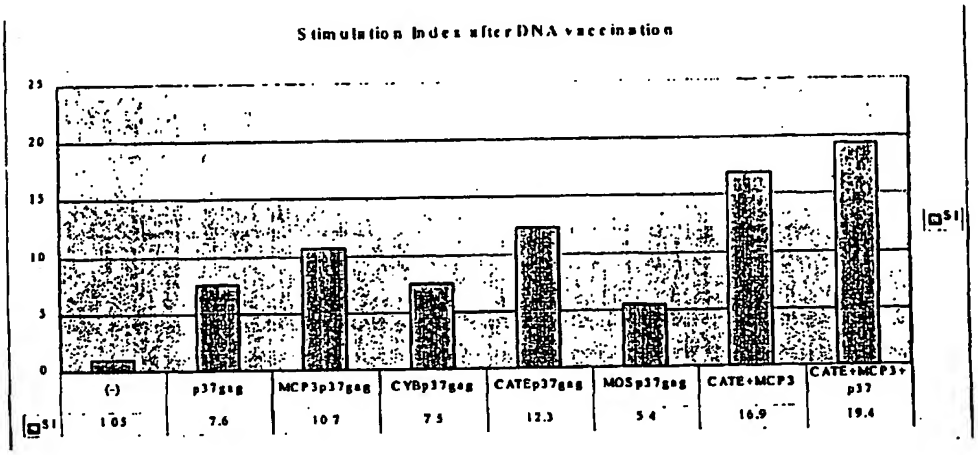

(57) Abstract: The invention relates to nucleic acids (such as DNA immunization plasmids), encoding fusion proteins containing a destabilizing amino acid sequence attached to an amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the destabilizing amino acid sequence. The invention also relates to nucleic acids encoding secreted fusion proteins, such as those containing chemokines or cytokines, and an attached amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased as a result of being attached to the secretory sequence. The invention also relates methods of increasing the immunogenicity of the encoded proteins for use as vaccines or in gene therapy.

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EXPRESSION VECTORS ABLE TO ELICIT IMPROVED IMMUNE RESPONSE AND METHODS OF USING SAME

I. TECHNICAL FIELD

The invention relates to nucleic acids (such as DNA immunization plasmids), encoding fusion proteins containing a destabilizing amino acid sequence attached to an amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the destabilizing amino acid sequence. The invention also relates to nucleic acids encoding secreted fusion proteins, such as those containing chemokines or cytokines, and an attached amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased as a result of being attached to the secretory sequence. The invention also relates methods of increasing the immunogenicity of the encoded proteins for use as vaccines or in gene therapy.

II. BACKGROUND

Cellular immune responses against human immunodeficiency virus type 1 (HIV-1) and the related simian immunodeficiency virus (SIV) have been shown to play an important role in controlling HIV-1 and SIV infection and in delaying disease progression. Containment of primary HIV-1 infection in infected individuals correlates with the emergence of virus-specific cytotoxic T-lymphocyte (CTL) responses (1, 2, 3). In chronically infected individuals, a high-frequency CTL response against HIV-1 is also correlated with a low viral load and slow disease progression (4, 5). An HIV-1-specific CTL response has also been demonstrated in certain highly exposed seronegative individuals (6, 7, 8). Also, strong HIV-specific proliferative responses, which may be critical for the maintenance of CTL responses, have been identified in long-term nonprogressors (9, 10).

HIV-1 Gag is one of the most conserved viral proteins. Broad, cross-clade CTL responses recognizing conserved epitopes in HIV-1 Gag have been detected in HIV-1 infected people (11, 12), and the development of a safe and effective HIV-1 vaccine may depend on the induction of effective CTL and/or T-helper responses against conserved HIV-1 proteins such as Gag. DNA vaccines have been shown to induce efficient cellular immune responses and protection against a variety of viral, bacterial, and parasitic pathogens in animal models. However, DNA vaccines that could induce potent cellular immune responses against HIV-1 Gag are not yet available.

We have recently demonstrated that by destroying inhibitory sequences in the coding region of HIV-1 gag, we could significantly increase Gag protein expression in primate as well as in mouse cells (13, 14, 15, 16) and dramatically enhance immune repsonse induced by a DNA vaccine (13). Since this new Gag expression vector is Rev/RRE-independent and species-independent, it provides a feasible approach to systematically evaluating the strategies that could lead to the maximum induction of cellular immune responses against HIV Gag molecules in animal models.

Intramuscular (i.m.) administration of a DNA vaccine represents a simple and effective means of inducing both humoral and cellular immune responses (17). There are three potential pathways reponsible for antigen presentation after i.m. injection of DNA. First, muscle cells could take up the DNA, express the encoded protein antigen, and present it to immune cells. Recent data suggest that this pathway is rather unlikely in vivo (18). Second, antigen presenting cells such as dendritic cells attracted to the site of injection may take up the DNA, express the encoded protein, and present it to T and B cells. Third, muscle cells may take up the DNA and express the protein antigen, with the antigen then being transmitted to dendritic cells for presentation. If the second possibility is the case, a protein that is synthesized and degraded in the cytoplasm of dendritic cells would be an excellent target for major histocompatibility complex (MHC) class I presentation and induction of CTL responses. Alternatively, if the third scenario were true, a protein synthesized in the muscle cells that could be targeted efficiently to dendritic cells would induce the best CTL response.

To distinguish among these different possibilities, three different forms of HIV-1 Gag DNA vaccine vectors were constructed and compared for the induction of immune responses. These different forms of Gag included (i) a standard Gag (St-Gag) (also called "WT" gag herein) that assembles into particles, which are efficiently released from cells and become surrounded by host-cell-derived lipid membrane acquired during virus budding; (ii) a cytoplasmic form of Gag (Cy-Gag) that fails to target the plasma membrane and therefore remains in the cytoplasm; and (iii) a secreted form of Gag (Sc-Gag) that is synthesized on the cytoplasmic face of the rough endoplasmic reticulum (ER), transported through the ER and Golgi apparatus, and released as a secreted protein (i.e., not surrounded by a lipid membrane) (19). (Mutant Gag proteins that are not targeted efficiently to the plasma membrane and remain primarily in the cytoplasm were created by destroying the myristylation signal of HIV-1 Gag. Sc-Gag molecules were created by the addition of the t-PA signal peptide sequence to the N terminus of the HIV-1 Gag molecule. This sequence provides a signal for translocation of the secreted protein into the lumen of the ER, for transport through the ER and Golgi apparatus, and for release in the form of Sc-Gag molecules.) (19).

In the study described above, the question of whether targeting HIV-1 Gag to various subcellular compartments could influence the induction of immune responses in DNA-immunized mice was addressed. The results demonstrated that targeting the HIV-1 Gag molecules to different subcellular compartments does indeed influence both the humoral and cellular immune responses that are elicited by i.m. DNA vaccination. Specifically, when these forms of Gag were administered to mice as a DNA vaccine, it was found that the DNA vector encoding the Sc-Gag generated better primary CTL and T-helper responses than did the DNA vector encoding Cy-Gag. Furthermore, the DNA vector encoding the Sc-Gag also generated a higher level of secondary CTL responses than did the DNA vector encoding Cy-Gag after DNA priming and recombinant vaccinia virus-Gag infection. Vaccinia virus titers were notably reduced in the ovaries of mice immunized with Gag DNA vaccine more than 125 days before infection, as compared to the titer in mice that received only the control DNA vector. These data indicated that $CD8^+$ T-cell memory elicited by DNA vaccination is functionally relevant and provides

protective immunity in this system. The DNA vector encoding the Sc-Gag provided better protection against recombinant vaccinia virus-Gag than did the DNA vector encoding Cy-Gag (19).

Another study has shown that altering the cellular location of glycoprotein D (gD) from bovine herpesvirus 1 by DNA vaccine modulates humoral immune response. Although both the secreted and cytosolic forms of gD induced an IgG2a antibody response, the secreted from of gD induced a stronger IgG1 response than IgG2a response (23). Similar results for Sc-Gag and Cy-Gag were observed in the study described above. On the other hand, St-Gag (also called "WT" gag herein), which is competent for forming virus-like particles, induced a predominantly IgG2a antibody repsonse. This latter data is consistent with the idea that location of antigens after DNA immunization could influence the type and potency of humoral immune responses.

Although DNA vaccines alone have been shown to protect against pathogenic challenges in small animals (24), their performance in primates has been generally disappointing. DNA vaccines, even with repeated boosting, induce only moderate immune responses when compared to live-attenuated virus or recombinant virus vaccines. However, recent studies have demonstrated that heterologuous priming-boosting immunization regimens using DNA plus recombinant modified vaccinia virus Ankara vectors can induce strong cellular immune responses and protection against malaria in mice (25), (26) and SIVmac (27), (28) in monkey models. Although T-cell immune responses induced by DNA immunization are moderate, they are highly focused upon a few specific epitopes, because of the small number of other epitopes expressed by this antigen delivery system. A boost with a recombinant vaccinia virus expressing the same antigen presumably stimulates this population of primed memory T cells. Our data showed that pSc-GAG induced higher memory T-cell responses than other Gag expression vectors as measured by *ex vivo* CTL activity, higher number of $CD8^+$ IFN-γ-producing cells after stimulation with MHC class I-restricted HIV-1 Gag-specific peptide, and greater protection against recombinant vaccinia virus-Gag infection (19). These Gag expression vectors may be useful for further evaluation of heterologous priming and boosting with DNA plus viral vector in inducing protective cellular immune

responses. Similar strategies could be considered for nonhuman primate models where SIV or simian/human immunodeficiency virus challenge can be evaluated.

There have been several reports regarding the use of t-PA signal peptides in DNA vaccines. In the case of HIV-1 Env DNA vaccine (20), replacing the authentic signal peptide of gp160 with that of t-PA was intended to overcome the Rev/RRE requirement for Env protein expression (21). Replacing the signal peptide sequences of mycobacterial proteins with that of t-PA in DNA vectors has been shown to correlate with more protection against tuberculous challenge in mice, although CTL responses were not measured (22). DNA vectors containing fusion of t-PA peptide with *Plasmodium vivax* antigens did not significantly increase antibody production in mice, and cellular immune responses were not evaluated (39). Whether the t-PA signal peptide can enhance the induction of immune responses for cytoplasmic antigens in general by means of a DNA vaccine strategy requires further investigation.

Other reports, concerning potential cancer vaccines, have demonstrated that active immunizations of human patients with idiotypic vaccines elicited antigen-specific $CD8^+$ T-cell responses and antitumor effects (29). Several alternative preclinical strategies to develop vaccines have been previously reported, including fusion of tumor idiotype-derived single chain Fv ("scFv") with cytokines and immunogenic peptides such as interleukin ("IL")-2, IL-4 and granulocyte-macrophage colony-stimulating factor ("GM-CSF") (30, 31, 32). These fusions of scFv with cytokines, toxin fragments and viral peptides predominantly elicit a humoral response with undetectable activation of cell mediated immunity (see Table 2 of ref. 33). In a different approach, the model antigen is rendered immunogenic in mice by genetically fusing it to a chemokine moiety (33, 34, 35). Potent anti-tumor immunity was dependent on the generation of specific andi-idiotypic antibodies and both CD4+ and CD8+ T cells. These researchers hypothesize that administration of these vaccines as fusion proteins or naked DNA vaccines may allow efficient targeting of antigen-presenting cells in vivo. They also propose that chemokine fusion may represent a novel, general strategy for formulating clinically relevant antigens, such as existing or newly identified tumor and HIV antigens into vaccines for cancer and AIDS, respectively, which elicit potent $CD8^+$ T-cell immunity (33).

These researchers further state that with regard to HIV vaccine development, it has been shown that HIV cannot enter human cells unless it first binds to two types of cell-surface receptors: CD4 and chemokine receptors. The two major variantly tropic HIV viruses infect cells via CCR5 or CXCR4 co-receptors. Therefore, they state that one may envisage a chemokine fusion vaccine for HIV that would elicit not only T-cell and humoral responses against HIV, but possibly could interfere with the binding of HIV to the respective chemokine receptor, thus blocking infection. Finally, they also propose that their strategy may be further improved by modifying and mutating the chemokine moiety, or replacing it with the viral chemokine-like genes, which would reduce the risk of generation of autoantibodies against native chemokines.

Another strategy designed to enhance the induction of antigen-specific CTL responses involves targeting vaccine antigens directly into the MHC class I antigen-processing pathway, thereby providing more of the peptide epitopes that trigger the CTL response. A signal that targets proteins for proteasomal degradation is the assembly of a polyubiquitin chain attached to an accessible Lys residue in the target protein. One factor that influences the rate at which polyubiquitination occurs is the identity of the N-terminal residue of the target protein, as certain non-met N-termini target proteins for rapid degradation by the 26S proteasome. Townsend and others have shown that such "N-end rule" targeting of antigens can enhance their processing and presentation by the class I pathway in an *in vitro* setting. (See reference 36).

Proteins with non-Met N termini have been expressed in cells using fusion constructs in which the coding sequence of the target protein is fused in-frame to the C terminus of the coding sequence of ubiquitin. Ubiquitin is normally made in the cell as a polyprotein that is cleaved by ubiquitin hydrolases at the C-terminus of each ubiquitin subunit, giving rise to individual ubiquitin molecules. These same ubiquitin hydrolases will also cleave the ubiquitin target fusion protein at the C terminus of ubiquitin, exposing the N terminus of the target. In a recent study, Tobery and Siliciano generated ubiquitin fusions to HIV-1 nef with either Met or Arg at the N terminus of nef (UbMNef and UbRNef, respectively) (37). In *in vitro* experiments using vaccinia vectors to express UbMNef and UBRNef, it was

shown that although both vectors induced expression of comparable amounts of nef, the form of nef with an Arg residue at the N terminus and a much shorter half-life ($t_{1/2}$ = 15 min vs 10 h). Furthermore, immunization of mice with a vaccinia vector expressing the rapidly degraded UbRNef resulted in the induction of a more vigorous nef-specific CTL response than did immunization with a vaccinia vector expressing the stable UbMNef. Tobery and Siliciano conclude that augmenting nef-specific CTL responses by targeting the antigen for rapid cytoplasmic degradation represents an attractive strategy for vaccination against HIV (37).

In a more recent study, Tobery and Siliciano used the viral protein (HIV-1 nef) as a model tumor-associated antigen to evaluate the *in vivo* efficacy of the "N-end rule" targeting strategy for enhancing the induction of *de novo* CTL responses in mice. They state that their results suggest that the "N-end rule" targeting strategy can lead to an enhancement in the induction of CTL that is sufficient to confer protection against a lethal dose of antigen-expressing tumor cells (36).

In sum, to date, DNA vaccines expressing various antigens have been used to elicit immune responses. In many cases this response in polarized or suboptimal for practical vaccination purposes. The present invention demonstrates that combinations of DNA vaccines containing different forms of antigens, as well as administration of the DNA vaccines to different immunization sites, increase the immune response, and hence, are expected to provide practical DNA vaccination procedures.

III. SUMMARY OF THE INVENTION

The invention relates to nucleic acids (including, but not limited to, DNA immunization plasmids), encoding fusion proteins comprising a destabilizing amino acid sequence covalently attached to a heterologous amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the destabilizing amino acid sequence.

The invention also relates to nucleic acids encoding secreted fusion proteins comprising a secretory amino acid sequence, such as those containing chemokines or cytokines, covalently attached to a heterologous amino acid

sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the secretory amino acid sequence.

The invention also relates to products produced by the nucleic acids, e.g., mRNA, polypeptides, and viral particles, as well as vectors and vector systems comprising these nucleic acids. The invention also relates host cells comprising these nucleic acids, vectors, vector systems and/or their products.

The invention also relates to compositions comprising these nucleic acids, vectors, vector systems, products and/or host cells, and methods of using these compositions, either alone or in combination, to stimulate an improved immune response.

The invention also relates to methods of using the same or different nucleic acids, vectors, vector systems, products and/or host cells, or compositions thereof, in different sites to enhance the immune response.

The invention also relates to uses of these nucleic acids, vectors, vector systems, host cells and/or compositions to produce mRNA, polypeptides, and/or infectious viral particles, and/or to induce antibodies and/or cytotoxic and/or helper T lymphocytes.

The invention also relates to the use of these nucleic acids, vectors, vector systems, products and/or host cells, or compositions thereof, in gene therapy or as vaccines.

For example, the invention also relates to the use of these nucleic acid constructs, vectors, vector systems and or host cells for use in immunotherapy and immunoprophylaxis, e.g., as a vaccine, or in genetic therapy after expression, in mammals, preferably in humans. The nucleic acid constructs of the invention can include or be incorporated into lentiviral vectors, vaccinia vectors, adenovirus vectors, herpesvirus vectors or other expression vectors or they may also be directly injected into tissue cells resulting in efficient expression of the encoded protein or protein fragment. These constructs may also be used for *in-vivo* or *in-vitro* gene replacement, e.g., by homologous recombination with a target gene in-situ. They may also be used for transfecting cells *ex-vivo*.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Proliferative responses (shown as stimulation index, SI) in mice injected with the indicated vectors or combinations. Vectors are as described in the examples.

Figure 2. Proliferative responses (shown as stimulation index, SI) in mice injected two times with the indicated SIV expression plasmids or combinations. Together = injection of 3 DNAs at the same sites; 3 sites = injections of the same DNAs at separate sites. Vectors are as described in the examples.

Figure 3. Antibody response in monkeys. Two animals (#585, 587) were injected 4x with 5 mg intramuscularly ("i.m.") of MCP3p37gag expression vector. Two animals (#626, 628) were given the same DNA mucosally as liposome-DNA preparations. Titers plotted as reciprocal serum dilutions scoring positive in HIV p24 ELIZA tests.

Figure 4. Percent of IFNgamma + cells in CD8 population after in vitro stimulation with a gag peptide pool in macaques after three vaccinations with either WT+MCP3; WT+CATE; WT+MCP3+CATE; WT; or no vaccination ("Naïve"). (Note: WT means wild-type gag, also referred to as Standard gag (St-gag) herein; MCP3 means MCP3-gag fusions; CATE means ß-catenin-gag fusions).

Figure 5. Percent of IFNgamma + cells in CD8 population after in vitro stimulation with an env peptide pool in macaques after three vaccinations with either WT+MCP3; WT+CATE; WT+MCP3+CATE; WT; or no injection ("Naïve"). (Note: WT means wild type env; MCP3 means MCP3-env fusion; CATE means ß-catenin-env fusions).

Figure 6. Schematic diagram of the SIV envelope encoding vector CMVkan/R-R-SIVgp160CTE.

Figure 7. DNA sequence of the SIV envelope encoding vector CMVkan/R-R-SIVgp160CTE containing a mutated SIV env gene.

Figure 8. Nucleotide and amino acid sequence of MCP3-gp160 env (HIV) fusion.

Figure 9. Nucleotide and protein sequence of the beta-catenin-gp160 env (HIV) fusion.

Figure 10. Western blot of HIV env expression vectors. Optimized vectors for wild type sequence of gp160 (lanes 1, 2, 3) or the fusions to MCP-3 (lane 6, 9), tPA leader peptide (lane 4, 7) and beta-catenin (lane 5, 8) are shown. Transfections with purified plasmid DNA were performed in human 293 cells and either cell extracts (intracellular) or cell supernatants (extracellular) were loaded on SDS-acrylamide gels, blotted, and probed with anti-HIV env antibodies. The positions of gp120 and gp41 are shown. Open arrow indicates degradation products detected in lane 5. CTE and RTE indicates respective additional posttranscriptional control elements present in some vectors.

V. MODES FOR CARRYING OUT THE INVENTION

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only, and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate an embodiment of the invention and, together with the description, serve to explain the principles of the invention.

The invention relates to nucleic acids (including, but not limited to, DNA immunization plasmids), encoding fusion proteins comprising a destabilizing amino acid sequence covalently attached to a heterologous amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the destabilizing amino acid sequence.

The invention also relates to nucleic acids encoding secreted fusion proteins comprising a secretory amino acid sequence, such as those containing chemokines or cytokines, covalently attached to a heterologous amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the secretory amino acid sequence.

The invention relates to nucleic acids having sequences encoding fusion proteins containing destabilizing amino acid sequences which increase the immunogenicity of an attached amino acid sequence, and to methods of using compositions comprising these nucleic acids, or combinations thereof, to increase the immunogenicity of the encoded protein(s). This invention also relates to nucleic

acids encoding a fusion protein containing MCP-3 amino acid sequences and HIV gag or env, or SIV gag or env, and additional proteins related to vaccinations against non-tumor associated antigens, such as pathogen antigens. The invention also relates to methods of using different immunization sites to increase the immunogenicity of the encoded protein(s).

One aspect of the invention relates to a nucleic acid construct encoding a fusion protein comprising a destabilization sequence covalently linked to an amino acid sequence containing one or more disease-associated antigen. Preferred destabilization sequences are those which target the fusion protein to the ubiquitin proteosomal degradation pathway. More preferably, the destabilization sequence is present in the amino acid sequences selected from the group consisting of c-Myc aa2-120; Cyclin A aa13-91; Cyclin B aa13-91; IkBa aa20-45; β-Catenin aa19-44; c-Jun aa1-67; and c-Mos aa1-35, and functional fragments thereof.

In one embodiment, the invention relates to nucleic acids comprising sequences which encode polypeptides containing a destabilizing amino acid sequence which increases the immunogenicity of a covalently attached amino acid sequence containing a clinically relevant antigen, such as a disease associated antigen, as compared to its immunogenicity in the absence of the destabilizing amino acid sequence.

In another embodiment, the invention relates to nucleic acids encoding secreted fusion proteins, such as those containing immunostimulatory chemokines, such as MCP-3 or IP-10, or cytokines, such as GM-CSF, IL-4 or IL-2. In a preferred embodiment, the invention relates to fusion proteins containing MCP-3 amino acid sequences and viral antigens such as HIV gag and env or SIV gag or env.

The nucleic acid sequences of the constructs of the invention can be synthetic (e.g., synthesized by chemical synthesis), semi-synthetic (e.g., a combination of genomic DNA, cDNA, or PCR amplified DNA and synthetic DNA), or recombinantly produced. The nucleic acid sequences also may optionally not contain introns. The nucleic acid sequence encoding the destabilizing amino acid sequence is preferably linked in frame to the N - terminal of a nucleic acid sequence encoding one or more antigen(s) of interest, or immunogenic epitope(s) thereof.

These sequences may optionally be linked by another sequence encoding one or more linker amino acids.

In addition, nucleic acid sequences encoding more than one antigens of interest, may optionally be operably linked in frame or via an internal ribosomal entry site (IRES), e.g., from picornaviral RNA. An IRES will be used in circumstances that one wants to express two proteins (or antigens) from the same promoter. Using an IRES the expression of the two proteins is coordinated. A further polypeptide encoding sequence may also be present under the control of a separate promoter. Such a sequence may encode, for example, a selectable marker, or further antigen(s) of interest. Expression of this sequence may be constitutive; for example, in the case of a selectable marker this may be useful for selecting successfully transfected packaging cells, or packaging cells which are producing particularly high titers of vector particles. Alternatively or additionally, the selectable marker may be useful for selecting cells which have been successfully infected with nucleic acid sequence and have the sequence integrated into their own genome.

The constructs of the invention may also encode additional immunostimulation molecules, such as the chemokine MCP-3 exemplified herein, and functional fragments thereof. These immunostimulation molecules may be encoded by nucleic acid sequences as part of the fusion protein expression unit or may be encoded by nucleic acid sequences as part of a separate expression unit. These molecules may also be encoded by sequences present on different nucleic acid constructs, vectors, etc. Immunostimulatory molecules such as cytokines, chemokines or lymphokines are well known in the art. See, e.g., U.S. Patent 6,100,387 which is incorporated by reference herein. See, also, e.g., Biragyn and Kwack (1999) (ref. 34).

When HIV or SIV antigens are encoded, the nucleic acids of the invention may also contain Rev-independent fragments of genes which retain the desired function (e.g., for antigenicity of Gag or Pol, particle formation (Gag) or enzymatic activity (Pol)), or they may also contain Rev-independent variants which have been mutated so that the encoded protein loses a function that is unwanted in certain circumstances. In the latter case, for example, the gene may be modified to

encode mutations (at the amino acid level) in the active site of reverse transcriptase or integrase proteins to prevent reverse transcription or integration. Rev-independent fragments of the gag gene and env gene are described in U.S. Patent Nos. 5,972,596 and 5,965,726, which are incorporated by reference herein. See also, PCT/US00/34985 filed December 22, 2000 (published as WO 01/46408 on June 28, 2001) for the gag gene and Figures 6 and 7 herein for the SIV env gene.

The expression of the proteins encoded by these nucleic acid constructs or vectors after transfection into cells may be monitored at both the level of RNA and protein production. RNA levels are quantitated by methods known in the art, e.g., Northern blots, S1 mapping or PCR methods. Protein levels may also be quantitated by methods known in the art, e.g., western blot or ELISA or fluorescent detection methods. A fast non-radioactive ELISA protocol can be used to detect gag protein (DUPONT or COULTER gag antigen capture assay).

Various vectors are known in the art. See, e.g., U.S. Patent 6,100,387, which is incorporated by reference herein. Preferred vectors considered useful in gene therapy and/or as a vaccine vectors, are lentiviral having, depending on the desired circumstances,

a) no round of replication (i.e., a zero replication system)

b) one round of replication, or

c) a fully replicating system

Such vectors are described, e.g., in PCT/US00/34985 filed December 22, 2000 (published as WO 01/46408 on June 28, 2001); and U.S. Serial No. 09/872,733, filed June 1, 2001, which are incorporated by reference herein.

In a preferred embodiment, a HIV- or SIV- based lentiviral system useful in the invention comprises the following three components:

1) a packaging vector containing nucleic acid sequences encoding the elements necessary for vector packaging such as structural proteins (except for HIV *env*) and the enzymes required to generate vector particles, the packaging vector comprising at least a mutated Rev-independent HIV or SIV *gag/pol* gene;

2) a transfer vector containing genetic cis-acting sequences

necessary for the vector to infect the target cell and for transfer of the therapeutic or reporter or other gene(s) of interest, the transfer vector comprising the encapsidation signal and the gene(s) of interest or a cloning site for inserting the gene(s) of interest;

and

3) a vector containing sequences encoding an element necessary for targeting the viral particle to the intended recipient cell, preferably the gene encoding the G glycoprotein of the vesicular stomatis virus (VSV-G) or amphotrophic MuLV or lentiviral *envs*.

In such vectors, when the CMV promoter or other strong, high efficiency, promoter is used instead of the HIV-1 LTR promoter in the packaging vector, high expression of *gag, pol,* or *gag/pol* can be achieved in the total absence of any other viral protein. The exchange of the HIV-1 LTR promoter with other promoters is beneficial in the packaging vector or other vectors if constitutive expression is desirable and also for expression in mammalian cells other than human cells, such as mouse cells, in which the HIV-1 promoter is weak. In certain embodiments, the presence of heterologous promoters will also be desired in the transfer vector and the envelope encoding vector, when such vectors are used.

The antigens of interest, in particular, clinically relevant antigens, are chosen according to the effect sought to be achieved. Preferably, the antigen induces antibodies or helper T-cells or cytotoxic T-cells.

Amino acids, or antigens, of interest useful the nucleic acid constructs of the invention are described, e.g., in U.S. Patent 5,891,432, which is incorporated by reference herein (see, e.g., Col. 13, ln. 20 to Col. 17, ln. 67). These antigens include, but are not limited to, disease associated antigens such as tumor-associated antigens, autoimmune disease-associated antigens, infectious disease-associated antigens, viral antigens, parasitic antigens and bacterial antigens. Tumor associated antigens include, but are not limited to, p53 and mutants thereof, Ras and mutants thereof, a Bcr/Abl breakpoint peptide, HER-2/neu, HPV 2, E6, HPV E7, carcinoembryonic antigen, MUC-1, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-

2, N-acetylglucosaminyltransferase-V, p15, gp100, MART-1/MelanA, tyrosinase, TRP-1, beta-catenin, MUM-1 and CDK-4, N-acetylglucosaminyltransferase-V, p15, gp100, MART-1/MelanA, tyrosinase, TRP-1, beta-catenin, MUM-1 and CDK-4. HIV or SIV antigens include, but are not limited to Gag, Env, Pol, Nef, Vpr, Vpu, Vif Tat and Rev. In a preferred embodiment of the invention, the HIV Gag-Pol-Tat-Rev-Nef or Tat-Rev-Env-Nef antigens are linked together, but are not active as HIV components.

Nucleic acid constructs of the invention, as well as vectors, vector systems or viral particles containing such nucleic acid constructs, or the encoded proteins may be used for gene therapy *in vivo* (e.g., parenteral inoculation of high titer vector) or *ex vivo* (e.g., *in vitro* transduction of patient's cells followed by reinfusion into the patient of the transduced cells). These procedures are been already used in different approved gene therapy protocols.

One way of performing gene therapy is to extract cells from a patient, infect the extracted cells with a vector, such as a lentiviral vector, or a viral particle and reintroduce the cells back into the patient. A selectable marker may be used to provide a means for enriching for infected or transduced cells or positively selecting for only those cells which have been infected or transduced, before reintroducing the cells into the patient. This procedure may increase the chances of success of the therapy. Selectable markers may be for instance drug resistance genes, metabolic enzyme genes, or any other selectable markers known in the art. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, e.g., histidinol, puromycin, hygromycin, neomycin, methotrexate, etc., and cell surface markers.

However, it will be evident that for many gene therapy applications of vectors, such as lentiviral vectors, selection for expression of a marker gene may not be possible or necessary. Indeed expression of a selection marker, while convenient for *in vitro* studies, could be deleterious *in vivo* because of the inappropriate induction of cytotoxic T lymphocytes (CTLs) directed against the foreign marker protein. Also, it is possible that for *in vivo* applications, vectors without any internal promoters will be preferable. The presence of internal promoters can affect for example the transduction titres obtainable from a packaging

cell line and the stability of the integrated vector. Thus, single transcription unit vectors, which may be bi-cistronic or poly-cistronic, coding for one or two or more therapeutic genes, may be the preferred vector designed for use *in vivo*. See, e.g., WO 98/17816.

Vaccines and pharmaceutical compositions comprising at least one of the nucleic acid sequences, polypeptides, viral particles, vectors, vector systems, or transduced or transfected host cells of the invention and a physiologically acceptable carrier are also part of the invention.

As used herein, the term "transduction" generally refers to the transfer of genetic material into the host via infection, e.g., in this case by the lentiviral vector. The term "transfection" generally refers to the transfer of isolated genetic material into cells via the use of specific transfection agents (e.g., calcium phosphate, DEAE Dextran, lipid formulations, gold particles, and other microparticles) that cross the cytoplasmic membrane and deliver some of the genetic material into the cell nucleus.

Pharmaceutical Compositions

The pharmaceutical compositions of the invention contain a pharmaceutically and/or therapeutically effective amount of at least one nucleic acid construct, polypeptide, vector, vector system, viral particle/virus stock, or host cell (i.e., agents) of the invention. If desired, the nucleic acid constructs, polypeptides, viral particles, vectors, vector systems, viral particle/virus stock, or host cells of the invention can be isolated and/or purified by methods known in the art.

In one embodiment of the invention, the effective amount of an agent of the invention per unit dose is an amount sufficient to cause the detectable expression of the antigen of interest. In another embodiment of the invention, the effective amount of agent per unit dose is an amount sufficient to prevent, treat or protect against deleterious effects (including severity, duration, or extent of symptoms) of the condition being treated. The effective amount of agent per unit dose depends, among other things, on the species of mammal inoculated, the body weight of the mammal and the chosen inoculation regimen, as is well known in the art. The dosage of the therapeutic agents which will be most suitable for

prophylaxis or treatment will also vary with the form of administration, the particular agent chosen and the physiological characteristics of the particular patient under treatment. The dose is administered at least once. Subsequent doses may be administered as indicated.

To monitor the response of individuals administered the compositions of the invention, mRNA or protein expression levels may be determined. In many instances it will be sufficient to assess the expression level in serum or plasma obtained from such an individual. Decisions as to whether to administer another dose or to change the amount of the composition administered to the individual may be at least partially based on the expression levels.

The term "unit dose" as it pertains to the inocula refers to physically discrete units suitable as unitary dosages for mammals, each unit containing a predetermined quantity of active material (e.g., nucleic acid, virus stock or host cell) calculated to produce the desired effect in association with the required diluent. The titers of the virus stocks to be administered to a cell or animal will depend on the application and on type of delivery (e.g., *in vivo* or *ex vivo*). The virus stocks can be concentrated using methods such as centrifugation. The titers to be administered *ex vivo* are preferably in the range of 0.001 to 1 infectious unit /cell. Another method of generating viral stocks is to cocultivate stable cell lines expressing the virus with the target cells. This method has been used to achieve better results when using traditional retroviral vectors because the cells can be infected over a longer period of time and they have the chance to be infected with multiple copies of the vector.

For *in vivo* administration of nucleic acid constructs, vectors, vector systems, virus stocks, or cells which have been transduced or transfected *ex vivo*, the dose is to be determined by dose escalation, with the upper dose being limited by the onset of unacceptable adverse effects. Preliminary starting doses may be extrapolated from experiments using lentiviral vectors in animal models, by methods known in the art, or may be extrapolated from comparisons with known retroviral (e.g., adenoviral) doses. Generally, small dosages will be used initially and, if necessary, will be increased by small increments until the optimum effect under the circumstances is reached. Exemplary dosages are within the range of 10^8 up to approximately 5×10^{15} particles.

For vaccinations DNA will be administered either IM in PBS as previously described in liposomes, by intradermal inoculation, electro-injection or other methods. As example, 5 mg per dose IM in macaques (DNA at 1mg/ml) injected at several different sites was found to produce a good immune response.

Inocula are typically prepared as a solution in a physiologically acceptable carrier such as saline, phosphate-buffered saline and the like to form an aqueous pharmaceutical composition.

The agents of the invention are generally administered with a physiologically acceptable carrier or vehicle therefor. A physiologically acceptable carrier is one that does not cause an adverse physical reaction upon administration and one in which the nucleic acids or other agents of the invention are sufficiently soluble to retain their activity to deliver a pharmaceutically or therapeutically effective amount of the compound. The pharmaceutically or therapeutically effective amount and method of administration of an agent of the invention may vary based on the individual patient, the indication being treated and other criteria evident to one of ordinary skill in the art. A nucleic acid construct of the invention is preferably present in an amount which is capable of expressing the encoded protein in an amount which is effective to induce antibodies and/or cytotoxic and/or helper-inducer T lymphocytes. A therapeutically effective amount of a nucleic acid of the invention is one sufficient to prevent, or attenuate the severity, extent or duration of the deleterious effects of the condition being treated without causing significant adverse side effects. The route(s) of administration useful in a particular application are apparent to one or ordinary skill in the art.

Routes of administration of the agents of the invention include, but are not limited to, parenteral, and direct injection into an affected site. Parenteral routes of administration include but are not limited to intravenous, intramuscular, intraperitoneal and subcutaneous. The route of administration of the agents of the invention is typically parenteral and is preferably into the bone marrow, into the CSF intramuscular, subcutaneous, intradermal, intraocular, intracranial, intranasal, and the like. See, e.g., WO 99/04026 for examples of formulations and routes of administration.

The present invention includes compositions of the agents described above, suitable for parenteral administration including, but not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for nasal, intravenous, intramuscular, intraperitoneal, subcutaneous or direct injection into a joint or other area.

In providing the agents of the present invention to a recipient mammal, preferably a human, the dosage administered will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical history and the like.

The administration of the pharmaceutical compositions of the invention may be for either "prophylactic" or "therapeutic" purpose. When provided prophylactically, the compositions are provided in advance of any symptom. The prophylactic administration of the composition serves to prevent or ameliorate any subsequent deleterious effects (including severity, duration, or extent of symptoms) of the condition being treated. When provided therapeutically, the composition is provided at (or shortly after) the onset of a symptom of the condition being treated.

For all therapeutic, prophylactic and diagnostic uses, one or more of the agents of the invention, as well as antibodies and other necessary reagents and appropriate devices and accessories, may be provided in kit form so as to be readily available and easily used.

Where immunoassays are involved, such kits may contain a solid support, such as a membrane (e.g., nitrocellulose), a bead, sphere, test tube, rod, and so forth, to which a receptor such as an antibody specific for the target molecule will bind. Such kits can also include a second receptor, such as a labeled antibody. Such kits can be used for sandwich assays to detect toxins. Kits for competitive assays are also envisioned.

VI. INDUSTRIAL APPLICABILITY

The nucleic acids of this invention can be expressed in the native host cell or organism or in a different cell or organism. The mutated genes can be

introduced into a vector such as a plasmid, cosmid, phage, virus or mini-chromosome and inserted into a host cell or organism by methods well known in the art. In general, the constructs can be utilized in any cell, either eukaryotic or prokaryotic, including mammalian cells (e.g., human (e.g., HeLa), monkey (e.g., Cos), rabbit (e.g., rabbit reticulocytes), rat, hamster (e.g., CHO and baby hamster kidney cells) or mouse cells (e.g., L cells), plant cells, yeast cells, insect cells or bacterial cells (e.g., E. coli). The vectors which can be utilized to clone and/or express nucleic acid sequences of the invention are the vectors which are capable of replicating and/or expressing the coding sequences in the host cell in which the coding sequences are desired to be replicated and/or expressed. See, e.g., F. Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience (1992) and Sambrook et al. (1989) for examples of appropriate vectors for various types of host cells. The native promoters for such coding sequences can be replaced with strong promoters compatible with the host into which the coding sequences are inserted. These promoters may be inducible. The host cells containing these coding sequences can be used to express large amounts of the protein useful in enzyme preparations, pharmaceuticals, diagnostic reagents, vaccines and therapeutics.

The constructs of the invention may also be used for in-vivo or in-vitro gene therapy. For example, a construct of the invention will produce an mRNA in situ to ultimately increase the amount of polypeptide expressed. Such polypeptides include viral antigens and/or cellular antigens. Such a constructs, and their expression products, are expected to be useful, for example, in the development of a vaccine and/or genetic therapy.

The constructs and/or products made by using constructs encoding antigens of interest could be used, for example, in the production of diagnostic reagents, vaccines and therapies for diseases, such as AIDS and AIDS-related diseases.

For example, vectors expressing high levels of Gag can be used in immunotherapy and immunoprophylaxis, after expression in humans. Such vectors include retroviral vectors and also include direct injection of DNA into muscle cells or other receptive cells, resulting in the efficient expression of gag, using the

technology described, for example, in Wolff et al., Science 247:1465-1468 (1990), Wolff et al., Human Molecular Genetics 1(6):363-369 (1992) and Ulmer et al., Science 259:1745-1749 (1993). Further, the gag constructs could be used in transdominant inhibition of HIV expression after the introduction into humans. For this application, for example, appropriate vectors or DNA molecules expressing high levels of $p55^{gag}$ or $p37^{gag}$ would be modified to generate transdominant gag mutants, as described, for example, in Trono et al., Cell 59:113-120 (1989). The vectors would be introduced into humans, resulting in the inhibition of HIV production due to the combined mechanisms of gag transdominant inhibition and of immunostimulation by the produced gag protein. In addition, the gag encoding constructs of the invention could be used in the generation of new retroviral vectors based on the expression of lentiviral gag proteins. Lentiviruses have unique characteristics that may allow the targeting and efficient infection of non-dividing cells. Similar applications are expected for vectors expressing high levels of env.

The following examples illustrate certain embodiments of the present invention, but should not be construed as limiting its scope in any way. Certain modifications and variations will be apparent to those skilled in the art from the teachings of the foregoing disclosure and the following examples, and these are intended to be encompassed by the spirit and scope of the invention.

EXAMPLE 1

Vectors

DNA vectors expressing antigens of HIV-1 or SIV are used in the examples herein.

Three different types of plasmids encoding forms of HIV Gag exemplified herein are as follows:

1) plasmids expressing full gag (p55) or parts of gag (p37) or gag and protease (p55gagpro). P55 produces gag particles that are partially released from the cell. P37 is partially released from the cell but does not form particles. P55gagpro also produces protease, therefore the gag is processed to form

p17, p24, p6 and p7;

2) plasmids expressing the chemokine MCP-3 fused to the N terminus of p55gag. Since MCP-3 is a secreted protein, the produced fusion protein is also secreted from the mammalian cells after the cleavage of the signal peptide; and

3) plasmids expressing fusions of gag to sequences conferring efficient proteasomal degratation.

Similar DNA expression vectors were produced for HIV env protein (see, e.g., Figures 8-9), as well as for SIV gag and env proteins. The HIV env plasmids were constructed based on a HIV clade B env sequence and tested for expression. Expression was high in the absence of Rev. (See Figure 10). Specific vectors, and combinations thereof, are described in more detail below. We also have variations of the vectors that do not contain linker amino acids, or contain fewer amino acids for CATENIN, etc, which are not specifically exemplified herein. Smaller fragments of the secretory sequences, or the destabilization sequence, than those exemplified herein, which maintain the desired function, are in some cases known to exist, or can be identified by routine experimentation. These sequences are also useful in the invention.

p37gag = HIV plasmid described previously

MCP3p37gag = as above, plus also contains also the leader sequence of ip10

The following is an example for MCP3p37gag :

The vector pCMVkanMCP3gagp37M1-10 expresses the following MCP3-gag fusion protein (SEQ ID NO: 1):

M N P S A A V I F C L I L L G L S G T Q
(IP10)
G I L D (linker)
M A Q P V G I N T S T T C C Y R F I N K K I P K
Q R L E S Y R R T T S S H C P R E A V I F K T K
L D K E I C A D P T Q K W V Q D F M K H L D K
K T Q T P K L (MCP-3)
A S A G A (linker)
G A R A S V L S G G E L D R W E K I R L R P G G
K K K Y K L K H I V W A S R E L E R F A V N P G
L L E T S E G C R Q I L G Q L Q P S L Q T G S E E
L R S L Y N T V A T L Y C V H Q R I E I K D T K
E A L D K I E E E Q N K S K K K A Q Q A A A D T
G H S N Q V S Q N Y P I V Q N I Q G Q M V H Q A
I S P R T L N A W V K V V E E K A F S P E V I P
M F S A L S E G A T P Q D L N T M L N T V G G H
Q A A M Q M L K E T I N E E A A E W D R V H P
V H A G P I A P G Q M R E P R G S D I A G T T S
T L Q E Q I G W M T N N P P I P V G E I Y K R W
I I L G L N K I V R M Y S P T S I L D I R Q G P K
E P F R D Y V D R F Y K T L R A E Q A S Q E V K
N W M T E T L L V Q N A N P D C K T I L K A L G
P A A T L E E M M T A C Q G V G G P G H K A R
V L E F • (p37gag HIV)

CYBp37gag = contains cyclin B destabilizing sequences

CATEp37gag = contains beta catenin destabilizing sequences

MOSp37gag = contains mos destabilizing sequences

SIVMCP3p39= as above for HIV

SIVCATEp39= as above for HIV

SIVgagDX is a Rev-independent SIV *gag* molecular clone. This vector is described in PCT/US00/34985 filed December 22, 2000 (published as WO 01/46408 on June 28, 2001), which is incorporated by reference herein. P39 denotes a DNA sequence encoding SIV Gag p39 (SIV p17 + p25). P57 denotes a DNA sequence encoding the complete SIV Gag p57.

"Gag" denotes DNA sequence encoding the Gag protein, which generates components of the virion core, "Pro" denotes "protease." The protease, reverse transcriptase, and integrase genes comprise the "pol" gene. In these constructs, "MCP3" denotes MCP-3 amino acids 33-109 linked to IP-10 secretory peptide refered supra (alternatively, it can be linked to its own natural secretory peptide or any other functional secretory signal such as the tPA signal mentioned supra), "CYB" denotes Cyclin B amino acids 10-95, "MOS" denotes C-Mos amino acid 1-35 and "CATE" denotes β-catenin amino acids 18-47.

Cyclin B nucleic acid sequences and encoded amino acids used in the constructs exemplified herein:

ATGTCCAGTGATTTGGAGAATATTGACACAGGAGT
TAATTCTAAAGTTAAGAGTCATGTGACTATTAGGC
GAACTGTTTTAGAAGAAATTGGAAATAGAGTTAC
AACCAGAGCAGCACAAGTAGCTAAGAAAGCTCAG
AACACCAAAGTTCCAGTTCAACCCACCAAAACAA
CAAATGTCAACAAACAACTGAAACCTACTGCTTCT
GTCAAACCAGTACAGATGGAAAAGTTGGCTCCAA
AGGGTCCTTCTCCCACACCTGTCGACAGAGAGATG
GGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAAT
TAGATCGATGGGAAAAAATTCGGTTAAGGCCAGG
GGGAAAGAAGAAGTACAAGCTAAAGCACATCGTA
TG (SEQ ID NO: 2)

MetSerSerAspLeuGluAsnIleAspThrGlyValAsnSerLysVal
LysSerHisValThrIleArgArgThrValLeuGluGluIleGlyAsnAr
gValThrThrArgAlaAlaGlnValAlaLysLysAlaGlnAsnThrLy
sValProValGlnProThrLysThrThrAsnValAsnLysGlnLeuLy

sProThrAlaSerValLysProValGlnMetGluLysLeuAlaProLys
GlyProSerProThrProValAspArgGlu (SEQ ID NO: 3)

c-Mos nucleic acid sequences and encoded amino acids used in the constructs exemplified herein:

ATGCCCGATCCCCTGGTCGACAGAGAG (SEQ ID NO: 4)

MetProAspProLeuValAspArgGlu (SEQ ID NO: 5)

EXAMPLE 2

Construction of Vectors

In order to design "Gag-destabilized" constructs, a literature search for characterized sequences able to target proteins to the ubiquitin-proteasome degradation pathway gave the following, not necessarily representative, list:

c-Myc	aa2-120
Cyclin A	aa13-91
Cyclin B	aa13-91 *we used 10-95 in vectors in examples herein
IkBa	aa20-45
b-Catenin	aa19-44 *we used 18-47 in vectors in examples herein
c-Jun	aa1-67
c-Mos	aa1-35

We cloned a subset of those degradation sequences from Jurkat cDNA, namely the signals from cyclin B, β-catenin, and c-Mos, using PCR. Both cyclin and catenin primers gave fragments of the expected length, that were cut and cloned into the SalI site of the vectors pCMV37(M1-10)kan, or pCMV55(M1-10)kan, and (Bam version) into the BamHI site of pFREDlacZ. (The p37 and p55 plasmids have the same p37 and p55 sequences disclosed in the patents containing INS- gag sequences (see, e.g., U.S. Patent No. 5,972,596 and U.S. Patent No. 5,965,726, which are incorporated by reference herein) but they have a different plasmid backbone expressing kanamycin. pFREDlacZ contains the IE CMV promoter expressing beta galactosidase of E coli.)

The corresponding plasmids are called:

pCMV37(M1-10)kan with cyclin B sequence in SalI site	**pS194**
pCMV37(M1-10)kan with β-catenin sequence in SalI site	**pS195**
pCMV55(M1-10)kan with cyclin B sequence in SalI site	**pS199**
pCMV55(M1-10)kan with β-catenin sequence in SalI site	**pS200**
pFREDlacZ with cyclin B sequence in BamHI site	**pS201**
pFREDlacZ with β-catenin sequence in BamHI site	**pS202**

In the case of Mos, the degradation signal consists of five N-terminal amino acids and a lysine approximately 30 amino acids away. A similarly located lysine is present in HIV gag, but not in lacZ. For that reason, oligos covering all five destabilizing amino acids were synthesized (both chains), annealed, and linked to the N-terminus of gag, but not lacZ. There were three versions of MOS sequence:

MOSN5wtUP & MOSN5wtDN	has serine shown to cause degradation when phosphorylated
MOSN5aspUP & MOSN5aspDN	has Asp for Ser substitution, mimicking phosphorylation for constitutive action
MOSN5argUP & MOSN5argDN	has Arg for Ser substitution, allegedly making degradation signal inactive

Out of six plasmids planned, we only examined the following:

pS191 having pCMV37(M1-10)kan with the wild type ("WT") Mos sequence, but the insert is longer than intended, with an additional copy of the synthetic sequence in reverse;

pS192 having pCMV37(M1-10)kan with "Asp" Mos sequence in the SalI site; and

pS197 with pCMV55(M1-10)kan with "Asp" Mos sequence in the SalI site.

EXAMPLE 3

Preliminary Characterization
of the Degradation Signals in the Vectors

The following experiments were conducted for preliminary characterization of the degradation signals in the nucleic acid constructs described above.

β-Galactosidase activity was measured in transiently transfected HeLa and 293 cells after transfection with either pFREDlacZ or its cyclin B or β-catenin- modified versions (pS201 & 202). Apparent loss of the lacZ activity was interpreted as being indicative of ubiquitination signal-induced protein degradation.

With modified Gag the following experiments were done to confirm that degradation signals work in the gag context as well. First, p24-gag was measured by ELISA in cellular extracts and supernatants of cells transfected with the modified Gag constructs. Although we obtained evidence of destabilization, in several cases this experiment measuring the total level of p24 antigen was inconclusive. This was probably because, as shown previously, fragments of gag can still score positive in the antigen capture assay procedure. Therefore we looked into how intact the produced proteins were.

Protein extracts of HeLa or 293 cells transiently transfected with different gag plasmids were run on acrylamide tris-glycine gel, transferred to Immobilon P membrane and stained with anti-HIV antibodies to reveal Gag. These experiments did not show any signs of degradation in HeLa cells, however 293 cells transformed with the cyclin or β-catenin-modified versions of Gag clearly demonstrated the presence of prominent Gag-stained bands of molecular weight smaller than the full-length modified Gag. Such non-full length bands were not observed with the wild type Gag-transfected cells. These finding is consistent with the signal-induced Gag degradation.

To further examine whether the N-terminal modifications induce Gag degradation, we conducted pulse-chase experiments with transiently transfected 293 cells. One day after transfection the cells were incubated in methionine-free medium to exhaust cellular pools, labeled with ^{35}S-methionine in the same medium, and

chased by adding 1000-fold excess of the cold methionine. Two experiments have been done. One with ~1 hour pulse and 12 hours chase, and another with 30 min pulse and 1.5 h chase. The experiments showed that the modified Gag degrades more rapidly than the wild type Gag. Both cyclin B and β-catenin-derived signals worked in destabilizing Gag to a similar extent. Additional experiments were performed with the env constructs-beta catenin fusions, and verified that the fusions were much more unstable after expression in human cells.

EXAMPLE 4

Proliferative Responses of Vectors And Combinations of Vectors

These vectors were tested for protein expression in vitro after transfections in mammalian cells and for immunogenicity in mice and primates (macaques).

Methods:

DNA was purified using the Qiagen endotoxin free DNA purification kit. Endotoxin levels were routinely measured and were very low (kinetic-QCL test, Bio-Whittaker gave approximately 1 endotoxin unit/mg of DNA in these preparations).

Mice were injected intramuscularly with 100 μg of DNA in 100 μl of PBS. Three injections of DNA were given at days 0 14 and 28. At day 35 mice were sacrificed and their splenocytes assayed for proliferation in the presence of the specific gag antigen. In addition, cytotoxic responses were evaluated by performing standard cytotoxicity assays. The antibody response of the vaccinated mice is also under evaluation using sera obtained from these animals.

For monkey experiments, 5 mg of MCP3gag HIV DNA in 5 ml of phosphate buffered saline (PBS) were injected in several spots intramuscularly in Rhesus macaques, after the animals were sedated. Four injections were given at 0, 2, 4, and 8 weeks. The animals were followed by several assays to assess cellular and humoral immune response. Previous immunizations with gag p37M1-10,

described in our previous patent gave only low levels of antibodies. The previous gag construct stimulated cellular immunity well, but not antibodies.

Figure 1 shows the proliferative responses (shown as stimulation index, SI) in mice injected with the indicated vectors or combinations of the following vectors containing DNA sequences encoding HIV polypeptides, or polypeptide controls:

p37gag
MCP3p37gag
CYBp37gag
CATEp37gag
MOSp37gag = *we used WT Mos in the example herein
CATE+MCP3 =*2 constructs, see above; these are the same plasmids used alone or in combinations
CATE+MCP3+p37 = *3 constructs, see above

Figure 2 shows proliferative responses (shown as stimulation index, SI) in mice injected two times with the indicated SIV expression plasmids or combinations. Together = injection of 3 DNAs at the same sites; 3 sites = injections of the same DNAs at separate sites. When the "same sites" were used, all DNAs were mixed and injected at the same body sites in the muscle. When separate sites were used, the DNAs were kept separate and injected at anatomical sites that are separate. This happened every time we immunized the mice, i.e., the 3 DNAs were kept separate and injected at different sites from each other; and different sites of injection were used for each vaccination.

SIVgagDX
SIVMCP3p39
SIVCATEp39
MCP3+CATE+P57 (together)
MCP3+CATE+P57 (3 sites)

Figure 3 shows the antibody response in monkeys. Two animals (#585, 587) were injected 4x with 5 mg IM of MCP3p37gag expression vector. Two animals (#626, 628) were given the same DNA mucosally as liposome-DNA preparations. Titers plotted as reciprocal serum dilutions scoring positive in anti-HIV p24 Eliza tests.

RESULTS

We found that MCP-3 fusions to gag dramatically increased the immune response to gag, compared to the unmodified gag vectors (type 1 as described above), see figures. This property may be in part the result of more efficient gag secretion from the cells, since we have recently shown that secreted gag having the leader sequence of tPA was more efficient in secretion and immunogenicity (Qiu et al, J. Virol. 2000).

In addition, this effect may be mediated by the function of MCP-3 molecule. The magnitude of the response suggests additional effects of MCP-3, in agreement with the reported effects of MCP-3 in inducing immunogenicity against a tumor antigen. Intramuscular injection of this MCP3p37gag in macaques led to the production of high titer anti-gag antibodies. This was not the case with previously tested gag expression vectors, indicating that it is possible to elicit an efficient antibody response in primates by only DNA vaccination. In addition, these results suggest that improved immunogenicity in mice was a satisfactory method to predict increased immunogenicity in primates. We therefore tested several vectors and combinations of vectors in mice, in an effort to identify the best combinations for subsequent experiments in primates.

We also studied the expression and immunogenicity of vectors that direct the expressed HIV antigens towards proteasome degradation and efficient presentation on the cell surface via the MHC-I class of molecules. MHC-I -- restricted immunity is known to be important for anti-viral defenses. MHC-I display intracellularly produced short peptides on cell surface. A change in the composition of the peptides exposed by a cell, signals to the immune system that the cell is abnormal (e.g. virally infected) and should be destroyed. The MHC-I -- exposed peptides originate from proteasomal degradation of cellular proteins. We tested the

hypothesis that supplying HIV antigens with strong additional ubiquitination signals targeting it for proteasomal degradation would increase its chances for being processed for surface presentation.

We tested several ubiquitination signals identified within known proteins for conferring rapid degradation after linking them to the N-terminus of HIV Gag. In parallel, the same ubiquitination signals were fused to beta-galactosidase to check for degradation efficiency by the drop in its enzymatic activity. This assay showed that all selected signals enhanced beta-galactosidase degradation.

The most effective sequence identified by these experiments corresponds to amino acids 18-47 of beta-catenin, a protein involved in Wnt signaling and cell-cell adhesion, whose abundance is controlled by degradation.

30 aa of Beta-catenin (18-47):

R K A A V S H W Q Q Q S Y L D S G I H S G A T T T A P S L S (SEQ ID NO: 6)

Beta-catenin(18-47) added at the N terminus of HIV antigens with initiator AUG Met:

M R K A A V S H W Q Q Q S Y L D S G I H S G A T T T A P S L S (SEQ ID NO: 7)

Injecting mice with DNA constructs expressing either HIV-I Gag, or Gag fused with beta-catenin destabilizing domain showed that the latter construct was more immunogenic. Compared with Gag alone, beta-catenin-Gag fusion evoked higher HIV-specific proliferative responses, elevated CTL response, and higher level of CD8+ IFNgamma+ -secreting cells.

Direct comparisons with other destabilizing sequences showed an overal higher potency of beta-catenin-Gag fusion. Therefore, one surprising conclusion is that, although several sequences increased proteasome processing and protein destabilization, the beta-catenin sequences were much better in inducing an increased immune response. Since the practical outcome of these studies is

improved vaccination procedures, we propose the use of preferably the beta-catenin sequences identified here for use in targeting antigens for degradation.

Another important conclusion came from studies of combinations of vectors expressing different forms of antigens. It was found that combinations showed improved immunogenicity especially when injected in different sites on the same mouse, compared to a mix of DNA vectors injected in the same site.

We propose that different forms of the antigens trigger qualitatively different immune responses. Therefore, combinations of antigens applied at different sites and also at different times, may increase protective immune response. The results so far support the conclusion that using different forms of DNA sequentially or in combinations but applied at different sites may reproduce the good immunogenicity obtained with other prime-boost vaccine combinations. This will be a dramatic improvement over existing procedures for DNA vaccination in primates, which has been shown to be inefficient, especially for stimulating humoral immunity.

EXAMPLE 5

Immunogenicity of SIV Gag and SIV Env DNA Vectors in Macaques

On the basis of previous data suggesting that the modified forms of HIV and SIV antigens showed different immune responses after DNA vaccination, we studied the immunogenicity of three different DNA vaccine vectors for SIV gag and SIV env in 12 macaques. The DNAs used are shown in Table 1, below:

Table 1

SIV DNA Vectors

	gag	full name:	
1	p57gag	SIVgagDX	WT
3	MCP3gag	SIVMCP3p39	extracellular
5	CATEgag	SIVCATEp39	intra cellular
	env		
2	gp160env	pCMVkan/R-R-SIVgp160CTE	WT
4	MCP3env	pCMVkan/MCP3/SIVgp160CTE	extracellular
6	CATEenv	pCMVkan/CATE/SIVgp160CTE	intra cellular

The SIV gag vectors are the same as those used in the mice experiments described in the previous examples above. The SIVenv parent vector has been described in patent application serial no. 09/872,733, filed June 1, 2001, which is incorporated by reference herein, as an example of a vector with high levels of expression. The schematic diagram and sequence of this vector are set forth in Figures 6 and 7 herein, respectively. The MCP3 and CATE fusion vectors contain the same sequences of MCP3 and CATE described for the gag vectors.

Three groups of four naïve macaques (groups 1, 2, 3) were immunized intramuscularly with purified DNA preparations in PBS as shown in Table 2:

Table 2

DNA Immunization

week:	0	4	12	24
Group 1:	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4
Group 2:	1,2,5,6	1,2,5,6	1,2,5,6	1,2,5,6
Group 3:	1,2,3,4,5,6	1,2,3,4,5,6	1,2,3,4,5,6	1,2,3,4,5,6
Group 4:	5,6	5, 6	3, 4	3, 4
Group 5:	1, 2	1, 2	1, 2	1, 2

The animals were injected with the indicated DNAs. The total amount of DNA injected each time per animal was kept constant at 3 mg for gag and 3 mg for env. Animals were injected at different sites with the different DNAs. Injections were intramuscularly with the DNA delivered in PBS at 1 mg/ml. The sites of injections were anatomically separate for the different DNAs.

In addition, four animals (group 4) were immunized first with DNAs 5 and 6 (i.e., SIV CATE gag and SIV CATE env), and subsequently at weeks 12 and 24 with DNAs 3 and 4 (i.e., SIV MCP3 gag and SIV MCP3 env). Two animals in group5 received the DNAs expressing unmodified, wild-type antigens for gag and env (1 and 2). The animals in groups 4 and 5 had been previously exposed to HIV DNA, but they were naïve for SIV antigens, which was verified by immunological assays (Antibody measurements and lymphoproliferative responses

to specific antigen stimulation). Despite this, animals in groups 4 and 5 showed early responses to SIV DNA injection, indicating an anamnestic response to SIV antigens. Therefore, the experiment for groups 4 and 5 needs to be repeated with naïve animals for final conclusions.

At sequential times during vaccination blood samples were obtained and analyzed for the presence of antibodies, lymphoproliferative responses and cytotoxic T cells.

The antibody titers obtained for gag are as shown in Table 3. The reciprocal of the highest dilution scoring positive in Elisa assays is shown. Empty cells indicate antibody reactivity below 1:50 dilution.

These results showed that administration of MCP3gag vector is associated with strong antibody response, because 8/8 (100%) of animals receiving MCP3gag (in Groups 1 and 3) developed high gag antibodies. In contrast, 3/6 (50%) of animals not receiving MCP3gag (in Groups 2 and 5) developed antibodies.

The specific cytotoxic T cell responses against gag and env were evaluated by measuring the number of CD8 cells that produce intracellular IFNgamma or TNFalpha in the presence of gag or env synthetic peptide pools (overlapping 15mers). The values obtained after three DNA vaccinations are shown in Figures 4 and 5. It is interesting that the combination of three vectors increased the number of specific IFNgamma-producing cells upon peptide stimulation. It was concluded that the animals receiving all three forms of antigens showed increased antibody response without diminishing cellular immune response. Actually the cellular immune response also showed increased cellular immune response and the results showed statistical significant differences.

These data indicate the development of a more balanced immune response than previously anticipated by DNA vaccination in macaques, by the combination of different antigen forms.

Group 4 responses (not shown above) were also elevated (1.11% and 0.88% for gag and env, respectively), but this needs to be repeated by vaccinating naïve animals.

The mechanism of this increased immunogenicity by the combination of DNA vectors needs to be examined further. Expression and secretion of MCP-3-antigen chimeras may lead to increased protein levels that stimulate efficiently humoral immune responses. The combination of different antigen forms may also promote better activation and coordination of effector cells.

Table 3 shows SIV gag antibody response for all groups from the time of first immunization.

Table 3

Antibody Titers In Monkeys Vaccinated with SIV DNAs (Groups 1-5)

	animal#	week 0	3	4	6	8	12	13	14	24	25	
Group1 WT+MCP3	918L				50		50	800	3200	50	800	12
	919L									50	50	3
	921L				50					50	50	
	922L							800	3200	50	50	3
Group2 WT+CATE	920L							200	800	50	50	
	923L								200	50	3200	3
	924L											
	925L											
Group3 WT+MCP3 +CATE	926L							50	200	50	3200	3
	927L									50	50	
	928L							50	800	50	50	3
	929L							50	200	50	3200	3
Group4 CATE, then MCP3	585L		800	800	3200	3200	800	3200	800	800	3200	3
	587L			50	50			3200	3200	12800	3200	3
	626L		800	200	50				50	50	3200	3
	628L									50	50	3
Group5 WT	715L		50			800	200	200	200	50	50	3
	716L						800					

EXAMPLE 6

Use Of Nucleic Acids of the Invention In Immunoprophylaxis Or Immunotherapy

In postnatal gene therapy, new genetic information has been introduced into tissues by indirect means such as removing target cells from the body, infecting them with viral vectors carrying the new genetic information, and then reimplanting them into the body; or by direct means such as encapsulating formulations of DNA in liposomes; entrapping DNA in proteoliposomes containing viral envelope receptor proteins; calcium phosphate co-precipitating DNA; and coupling DNA to a polylysine-glycoprotein carrier complex. In addition, in vivo infectivity of cloned viral DNA sequences after direct intrahepatic injection with or without formation of calcium phosphate coprecipitates has also been described. mRNA sequences containing elements that enhance stability have also been shown to be efficiently translated in Xenopus laevis embryos, with the use of cationic lipid vesicles. See, e.g., J.A. Wolff, et al., Science 247:1465-1468 (1990) and references cited therein.

It has also been shown that injection of pure RNA or DNA directly into skeletal muscle results in significant expression of genes within the muscle cells. J.A. Wolff, et al., Science 247:1465-1468 (1990). Forcing RNA or DNA introduced into muscle cells by other means such as by particle-acceleration (N. -S. Yang, et al. Proc. Natl. Acad. Sci. USA 87:9568-9572 (1990); S.R. Williams et al., Proc. Natl. Acad. Sci. USA 88:2726-2730 (1991)) or by viral transduction or in vivo electorporation should also allow the DNA or RNA to be stably maintained and expressed. In the experiments reported in Wolff et al., RNA or DNA vectors were used to express reporter genes in mouse skeletal muscle cells, specifically cells of the quadriceps muscles. Protein expression was readily detected and no special delivery system was required for these effects. Polynucleotide expression was also obtained when the composition and volume of the injection fluid and the method of injection were modified from the described protocol. For example, reporter enzyme activity was reported to have been observed with 10 to 100 µl of hypotonic, isotonic, and hypertonic sucrose solutions, Opti-MEM, or sucrose solutions containing 2mM $CaCl_2$ and also to have been observed when the 10- to 100- µl injections were performed over 20 min. with a pump instead of within 1 min.

Enzymatic activity from the protein encoded by the reporter gene was also detected in abdominal muscle injected with the RNA or DNA vectors, indicating that other muscles can take up and express polynucleotides. Low amounts of reporter enzyme were also detected in other tissues (liver, spleen, skin, lung, brain and blood) injected with the RNA and DNA vectors. Intramuscularly injected plasmid DNA has also been demonstrated to be stably expressed in non-human primate muscle. S. Jiao et al., Hum. Gene Therapy 3:21-33 (1992).

It has been proposed that the direct transfer of genes into human muscle in situ may have several potential clinical applications. Muscle is potentially a suitable tissue for the heterologous expression of a transgene that would modify disease states in which muscle is not primarily involved, in addition to those in which it is. For example, muscle tissue could be used for the heterologous expression of proteins that can immunize, be secreted in the blood, or clear a circulating toxic metabolite. The use of RNA and a tissue that can be repetitively accessed might be useful for a reversible type of gene transfer,

administered much like conventional pharmaceutical treatments. See J.A. Wolff, et al., Science 247:1465-1468 (1990) and S. Jiao et al., Hum. Gene Therapy 3:21-33 (1992).

It had been proposed by J.A. Wolff et al., supra, that the intracellular expression of genes encoding antigens might provide alternative approaches to vaccine development. This hypothesis has been supported by a recent report that plasmid DNA encoding influenza A nucleoprotein injected into the quadriceps of BALB/c mice resulted in the generation of influenza A nucleoprotein-specific cytotoxic T lymphocytes (CTLs) and protection from a subsequent challenge with a heterologous strain of influenza A virus, as measured by decreased viral lung titers, inhibition of mass loss, and increased survival. J. B. Ulmer et al., Science 259:1745-1749 (1993).

Therefore, it appears that the direct injection of RNA or DNA vectors encoding the viral antigen can be used for endogenous expression of the antigen to generate the viral antigen for presentation to the immune system without the need for self-replicating agents or adjuvants, resulting in the generation of antigen-specific CTLs and protection from a subsequent challenge with a homologous or heterologous strain of virus.

CTLs in both mice and humans are capable of recognizing epitopes derived from conserved internal viral proteins and are thought to be important in the immune response against viruses. By recognition of epitopes from conserved viral proteins, CTLs may provide cross-strain protection. CTLs specific for conserved viral antigens can respond to different strains of virus, in contrast to antibodies, which are generally strain-specific.

Thus, direct injection of RNA or DNA encoding the viral antigen has the advantage of being without some of the limitations of direct peptide delivery or viral vectors. See J.A. Ulmer et al., supra, and the discussions and references therein). Furthermore, the generation of high-titer antibodies to expressed proteins after injection of DNA indicates that this may be a facile and effective means of making antibody-based vaccines targeted towards conserved or non-conserved antigens, either separately or in combination with CTL vaccines targeted towards conserved antigens. These may also be used with traditional peptide vaccines, for

the generation of combination vaccines. Furthermore, because protein expression is maintained after DNA injection, the persistence of B and T cell memory may be enhanced, thereby engendering long-lived humoral and cell-mediated immunity.

Vectors for the immunoprophylaxis or immunotherapy against HIV-1

In one embodiment of the invention, the nucleic acids of the invention will be inserted in expression vectors containing REV independent expression cassettes using a strong constitutive promoter such as CMV or RSV, or an inducible promoter such as HIV-1.

The vector will be introduced into animals or humans in a pharmaceutically acceptable carrier using one of several techniques such as injection of DNA directly into human tissues; electroporation (in vivo or ex vivo) or transfection of the DNA into primary human cells in culture (ex vivo), selection of cells for desired properties and reintroduction of such cells into the body, (said selection can be for the successful homologous recombination of the incoming DNA to an appropriate preselected genomic region); generation of infectious particles containing the *gag* gene, infection of cells *ex vivo* and reintroduction of such cells into the body; or direct infection by said particles in vivo.

Substantial levels of protein will be produced (and rapidly degraded in the situations where destabilization sequences are part of the encoded protein) leading to an efficient stimulation of the immune system.

In another embodiment of the invention, the described constructs will be modified to express mutated Gag proteins that are unable to participate in virus particle formation. It is expected that such Gag proteins will stimulate the immune system to the same extent as the wild-type Gag protein, but be unable to contribute to increased HIV-1 production. This modification should result in safer vectors for immunotherapy and immunophrophylaxis.

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vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells," *Proc. Natl Acad. Sci. USA.* 95, 11939-11944 (1998)

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Those skilled in the art will recognize that any gene encoding a mRNA containing an inhibitory/instability sequence or sequences can be modified in accordance with the exemplified methods of this invention or their functional equivalents.

Modifications of the above described modes for carrying out the invention that are obvious to those of skill in the fields of genetic engineering, virology, immunology, medicine, and related fields are intended to be within the scope of the following claims.

Every reference cited hereinbefore throughout the application is hereby incorporated by reference in its entirety.

WHAT IS CLAIMED IS:

1. A nucleic acid construct containing nucleotide sequences encoding a fusion protein comprising a destabilizing amino acid sequence covalently attached to a heterologous amino acid sequence of interest in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the destabilizing amino acid sequence and wherein the destabilizing amino acid sequence is present in the amino acid sequences selected from the group consisting of c-Myc aa2-120; Cyclin A aa13-91; Cyclin B 10-95; Cyclin B aa13-91; IkBa aa20-45; β-Catenin aa19-44; c-Jun aa1-67; and c-Mos aa1-35.

2. A nucleic acid construct of claim 1 wherein the amino acid sequence of interest is a disease associated antigen.

3. A nucleic acid construct of claim 1 wherein the destabilization sequence A nucleic acid construct of claim 1 wherein the destabilization sequence is selected from the group consisting of c-Mos aa1-35; cyclin B aa 10-95; β-catenin 19-44 and β-catenin 18-47.

4. The nucleic acid construct of claim 2 wherein the disease associated antigen is selected from the group consisting of tumor-associated antigen, autoimmune disease-associated antigen, infectious disease-associated antigen, viral antigen, parasitic antigen and bacterial antigen.

5. The nucleic acid of claim 4 wherein said viral antigen is HIV antigen.

6. The nucleic acid of claim 5 wherein said HIV antigen is selected from the group consisting of Gag, Env, Pol, Nef, Vpr, Vpu, Vif, Tat and Rev.

7. The nucleic acid of claim 6 wherein the disease associated antigens comprise antigenic fragments of HIV Gag-Pol-Tat-Rev-Nef or Tat-Rev-Env-Nef linked together, not necessarily in that order.

8. The nucleic acid of claim 4, wherein said autoimmune disease-associated antigen is a T cell receptor derived peptide.

9. A vector comprising the nucleic acid construct of claim 1.

10. A host cell comprising the nucleic acid construct of claim 1.

11. A pharmaceutical composition comprising a nucleic acid of claim 1 and a pharmaceutically acceptable carrier.

12. A method of stimulating the immune response against an amino acid sequence of interest, comprising administering to a mammal a sufficient amount of pharmaceutical composition of claim 11 to stimulate an immune response.

13. A method for inducing antibodies in a mammal comprising administering to a mammal a composition of claim 11, wherein said nucleic acid construct is present in an amount which is effective to induce said antibodies in said mammal.

14. A method for inducing cytotoxic and/or helper-inducer T lymphocytes in a mammal comprising administering to a mammal a composition of claim 11, wherein said nucleic acid construct is present in an amount which is effective to induce cytotoxic and/or helper-inducer T lymphocytes in said mammal.

15. A vaccine composition for inducing immunity in a mammal against HIV infection comprising a therapeutically effective amount of a nucleic acid construct of Claim 1 and a pharmaceutically acceptable carrier.

16. A method for inducing immunity against HIV infection in a mammal which comprises administering to a mammal a therapeutically effective amount of a vaccine composition according to claim 15.

17. A fusion polypeptide encoded by the nucleic acid construct of claim 1.

18. A viral particle comprising the nucleic acid construct of claim 1.

19. A pharmaceutical composition comprising the viral particle of claim 18.

20. A method of stimulating the immune response against a amino acid sequence of interest, comprising administering to a mammal a sufficient amount of pharmaceutical composition of claim 19 to stimulate an immune response.

21. A nucleic acid construct encoding a secreted fusion protein

comprising a chemokine MCP-3 secretory leader amino acid sequence covalently attached to a heterologous amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the secretory amino acid sequence.

22. A nucleic acid construct of claim 21 wherein the amino acid sequence of interest is a disease associated antigen.

23. A nucleic acid construct of claim 21 wherein the chemokine MCP-3 secretory leader sequence is MCP-3 amino acids 33-109 or 1-109.

24. A nucleic acid construct of claim 21 wherein the construct is selected from the group consisting of a (a) construct comprising a sequence encoding HIV p37 gag, a MCP-3 secretory leader sequence and a leader sequence of IP10 and (b) a construct comprising a sequence encoding SIV p39 gag, a MCP-3 secretory leader sequence and a leader sequence of IP10.

25. The nucleic acid construct of claim 22 wherein the disease associated antigen is selected from the group consisting of tumor-associated antigen, autoimmune disease-associated antigen, infectious disease-associated antigen, viral antigen, parasitic antigen and bacterial antigen.

26. The nucleic acid of claim 25 wherein said viral antigen is HIV antigen.

27. The nucleic acid of claim 26 wherein said HIV antigen is selected from the group consisting of Gag, Env, Pol, Nef, Vpr, Vpu, Vif, Tat and Rev.

28. The nucleic acid of claim 25 wherein the disease associated antigens comprise antigenic fragments of HIV Gag-Pol-Tat-Rev-Nef or Tat-Rev-Env-Nef linked together, not necessarily in that order.

29. The nucleic acid of claim 25, wherein said autoimmune disease-associated antigen is a T cell receptor derived peptide.

30. A vector comprising the nucleic acid construct of claim 21.

31. A host cell comprising the nucleic acid construct of claim 21.

32. A pharmaceutical composition comprising a nucleic acid of claim 21 and a pharmaceutically acceptable carrier.

33. A method of stimulating the immune response against an

amino acid sequence of interest, comprising administering to a mammal a sufficient amount of pharmaceutical composition of claim 32 to stimulate an immune response.

34. A method for inducing antibodies in a mammal comprising administering to a mammal a composition of claim 32, wherein said nucleic acid construct is present in an amount which is effective to induce said antibodies in said mammal.

35. A method for inducing cytotoxic and/or helper-inducer T lymphocytes in a mammal comprising administering to a mammal a composition of claim 32, wherein said nucleic acid construct is present in an amount which is effective to induce cytotoxic and/or helper-inducer T lymphocytes in said mammal.

36. A vaccine composition for inducing immunity in a mammal against HIV infection comprising a therapeutically effective amount of a nucleic acid construct of claim 21 and a pharmaceutically acceptable carrier.

37. A method for inducing immunity against HIV infection in a mammal which comprises administering to a mammal a therapeutically effective amount of a vaccine composition according to claim 36.

38. A fusion polypeptide encoded by the nucleic acid construct of claim 21.

39. A viral particle comprising the nucleic acid construct of claim 21.

40. A pharmaceutical composition comprising the viral particle of claim 39.

41. A method of stimulating the immune response against an amino acid sequence of interest, comprising administering to a mammal a sufficient amount of pharmaceutical composition of claim 40 to stimulate an immune response.

42. A composition comprising a one or more vectors expressing different forms of an antigen covalently linked to destabilizing or secreting moieties.

43. A composition of claim 42 where at least one vector comprises a nucleic acid construct containing nucleotide sequences encoding a fusion protein comprising a destabilizing amino acid sequence covalently attached

to a heterologous amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the destabilizing amino acid sequence, and at least one vector comprises a nucleic acid construct encoding a secreted fusion protein comprising a secretory amino acid sequence covalently attached to a heterologous amino acid sequence of interest, in which the immunogenicity of the amino acid sequence of interest is increased by the presence of the secretory amino acid sequence.

44. A method for inducing antibodies in a mammal comprising administering to a mammal a composition of claim 42, wherein said vectors are present in an amount which is effective to induce said antibodies in said mammal.

45. A method for inducing cytotoxic and/or helper-inducer T lymphocytes in a mammal comprising administering to a mammal a composition of claim 42, wherein said vectors are present in an amount which is effective to induce cytotoxic and/or helper-inducer T lymphocytes in said mammal.

46. A method of claim 44 or 45 comprising administering the composition to the same site.

47. The method of claim 46 wherein the vectors are administered at the same time.

48. The method of claim 46 wherein the vectors are administered at different times.

49. A method of claim 44 or 45 comprising administering the composition to different sites.

50. The method of claim 49 wherein the vectors are administered at the same time.

51. The method of claim 49 wherein the vectors are administered at different times.

52. A composition comprising the vectors comprosing nucleic acids which encode wt gag, MCP3gag, and B-CATEgag.

53. A composition comprising the vectors wt env, MCP3env, and B-CATEenv.

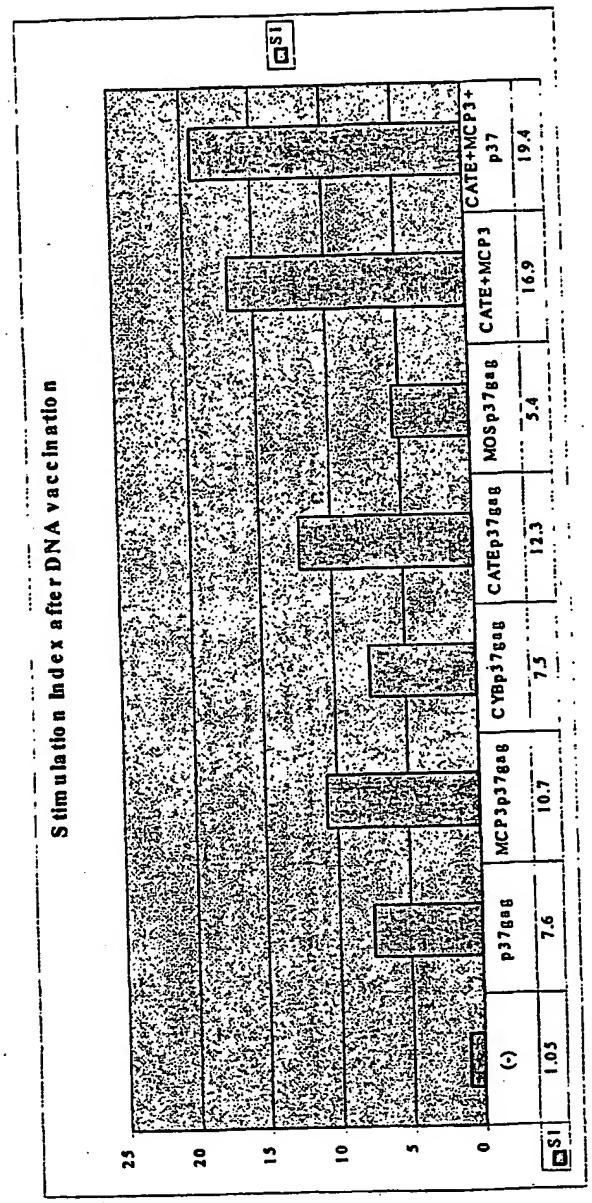

Stimulation Index after DNA vaccination
SI
25
20
15
10
5
0
(-)
1.05
p37gag
7.6
MCP3p37gag
10.7
CYBp37gag
7.5
CATEp37gag
12.3
MOSp37gag
5.4
CATE+MCP3
16.9
CATE+MCP3+ p37
19.4

FIGURE 1

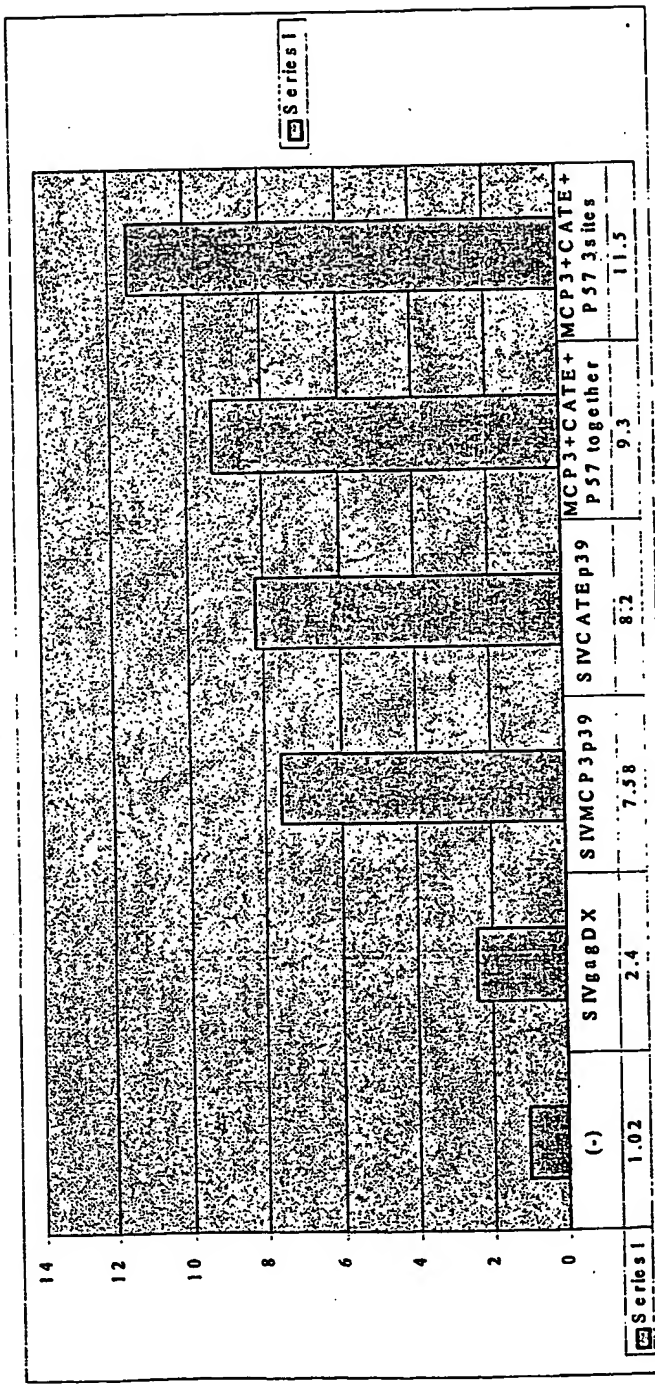

Series1
14
12
10
8
6
4
2
0
Series1
(-)
1.02
SIVgagDX
2.4
SIVMCP3p39
7.58
SIVCATEp39
8.2
MCP3+CATE+ P57 together
9.3
MCP3+CATE+ P57 3sites
11.5

FIGURE 2

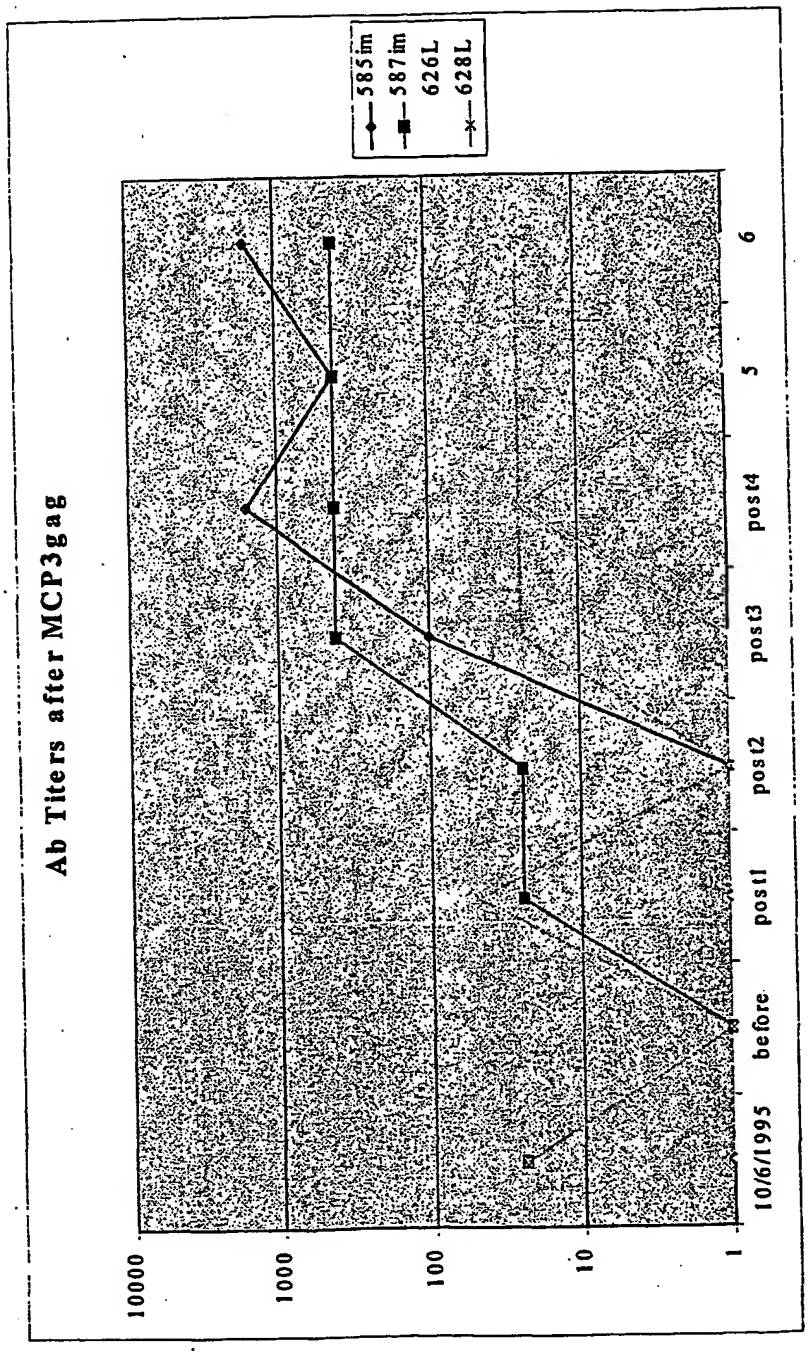

Ab Titers after MCP3gag
585im
587im
626L
628L
10000
1000
100
10
1
10/6/1995
before
post1
post2
post3
post4
5
6

FIGURE 3

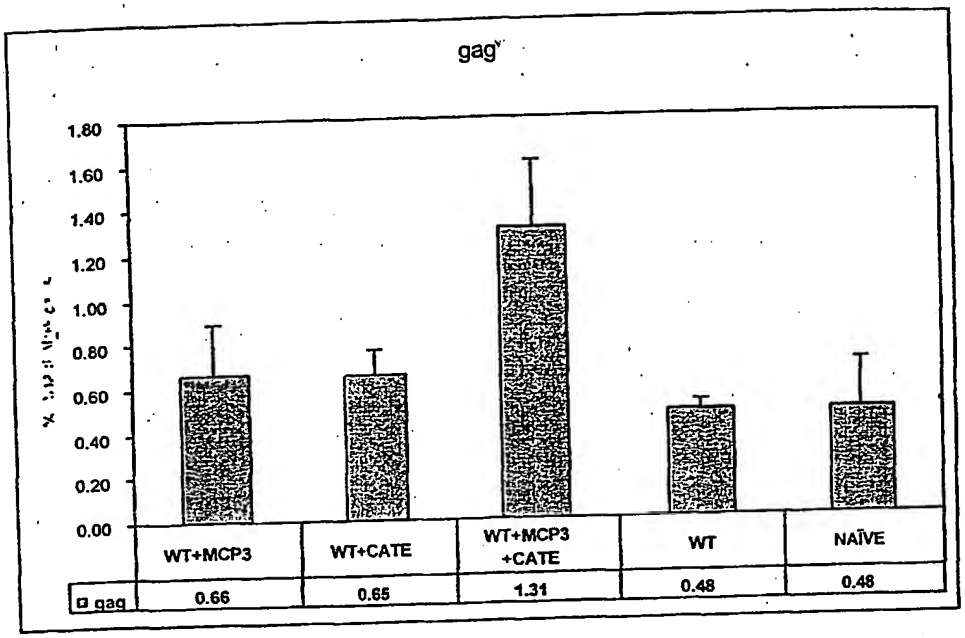

gag
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
WT+MCP3
WT+CATE
WT+MCP3 +CATE
WT
NAÏVE
gag
0.66
0.65
1.31
0.48
0.48

FIGURE 4

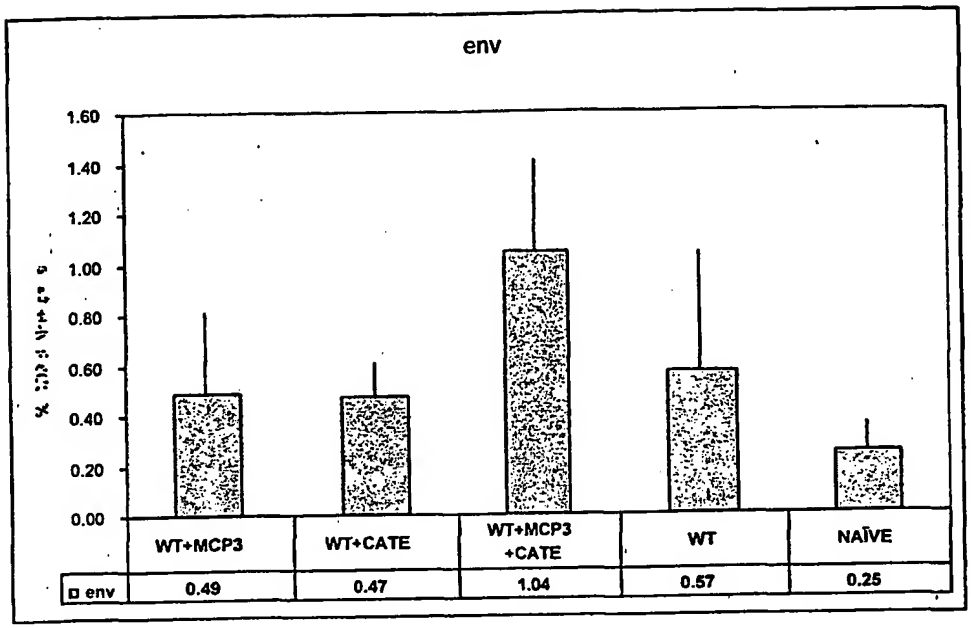

env
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
WT+MCP3
WT+CATE
WT+MCP3 +CATE
WT
NAÏVE
env
0.49
0.47
1.04
0.57
0.25

FIGURE 5

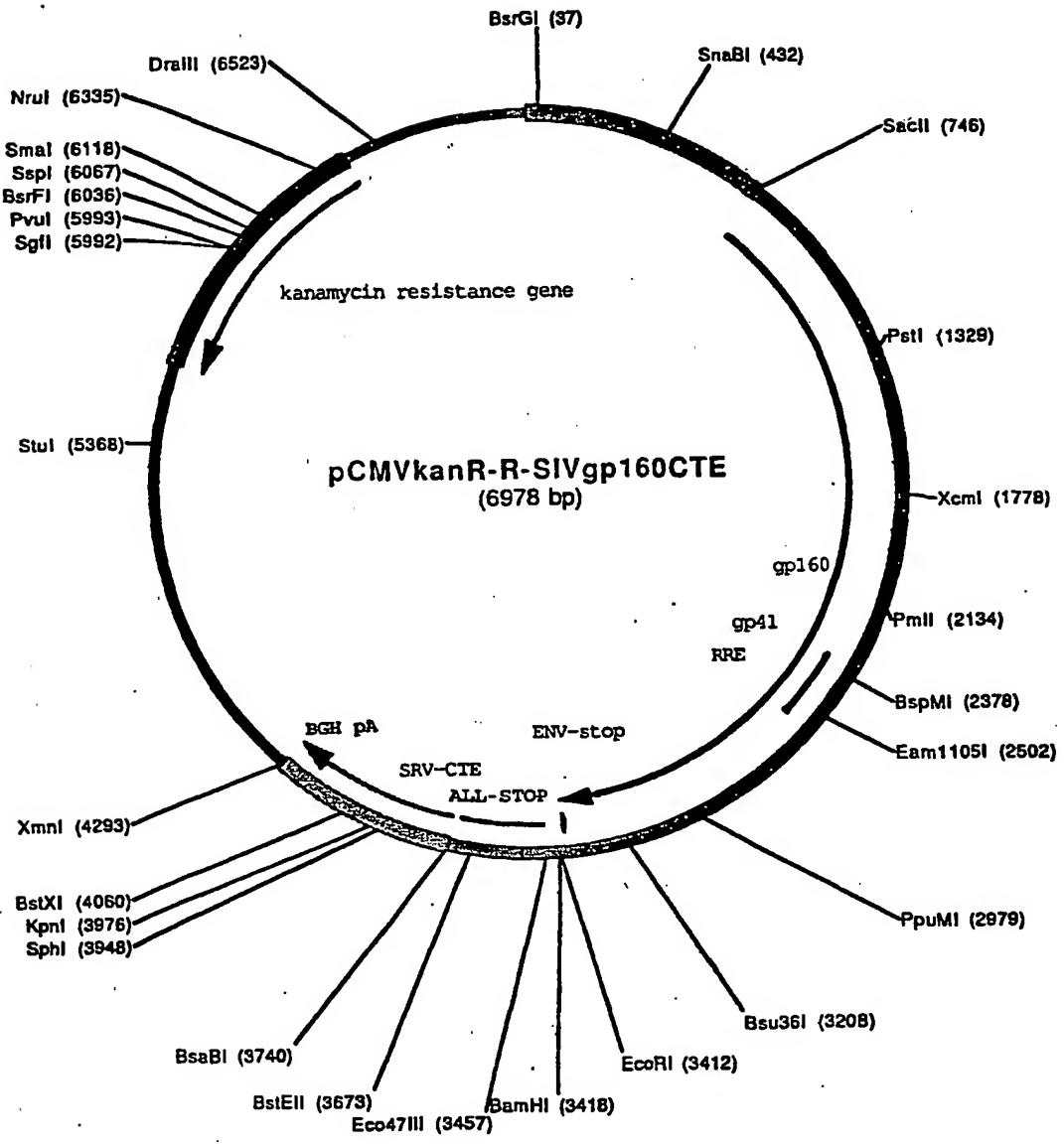

BsrGI (37)
SnaBI (432)
SacII (746)
PstI (1329)
XcmI (1778)
PmlI (2134)
BspMI (2378)
Eam1105I (2502)
PpuMI (2979)
Bsu36I (3208)
EcoRI (3412)
BamHI (3418)
Eco47III (3457)
BstEII (3673)
BsaBI (3740)
BstXI (4060)
KpnI (3976)
SphI (3948)
XmnI (4293)
StuI (5368)
NruI (6335)
DraIII (6523)
SmaI (6118)
SspI (6067)
BsrFI (6036)
PvuI (5993)
SgfI (5992)
kanamycin resistance gene
pCMVkanR-R-SIVgp160CTE
(6978 bp)
gp160
gp41
RRE
BGH pA
ENV-stop
SRV-CTE
ALL-STOP

FIGURE 6

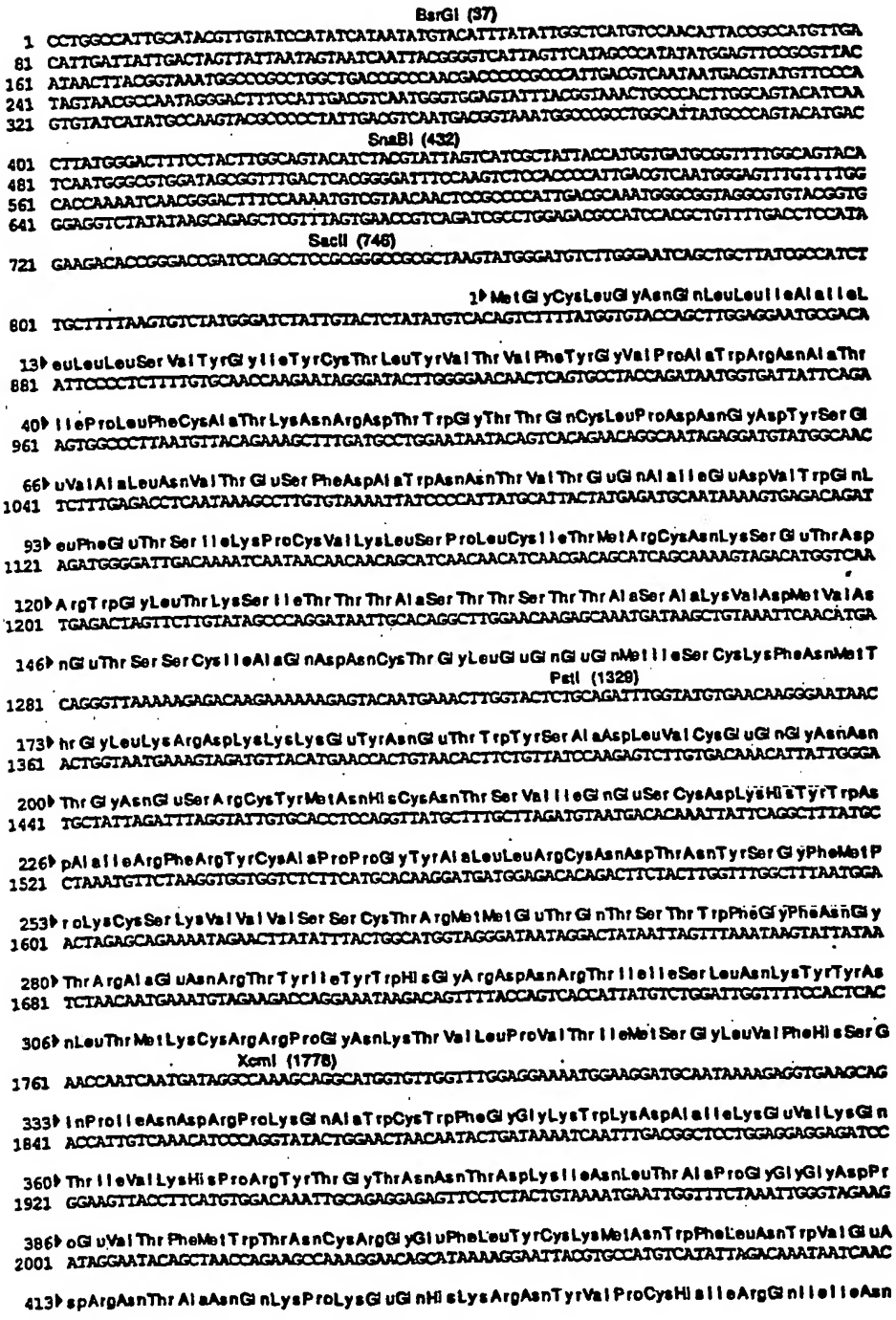


FIGURE 7-1

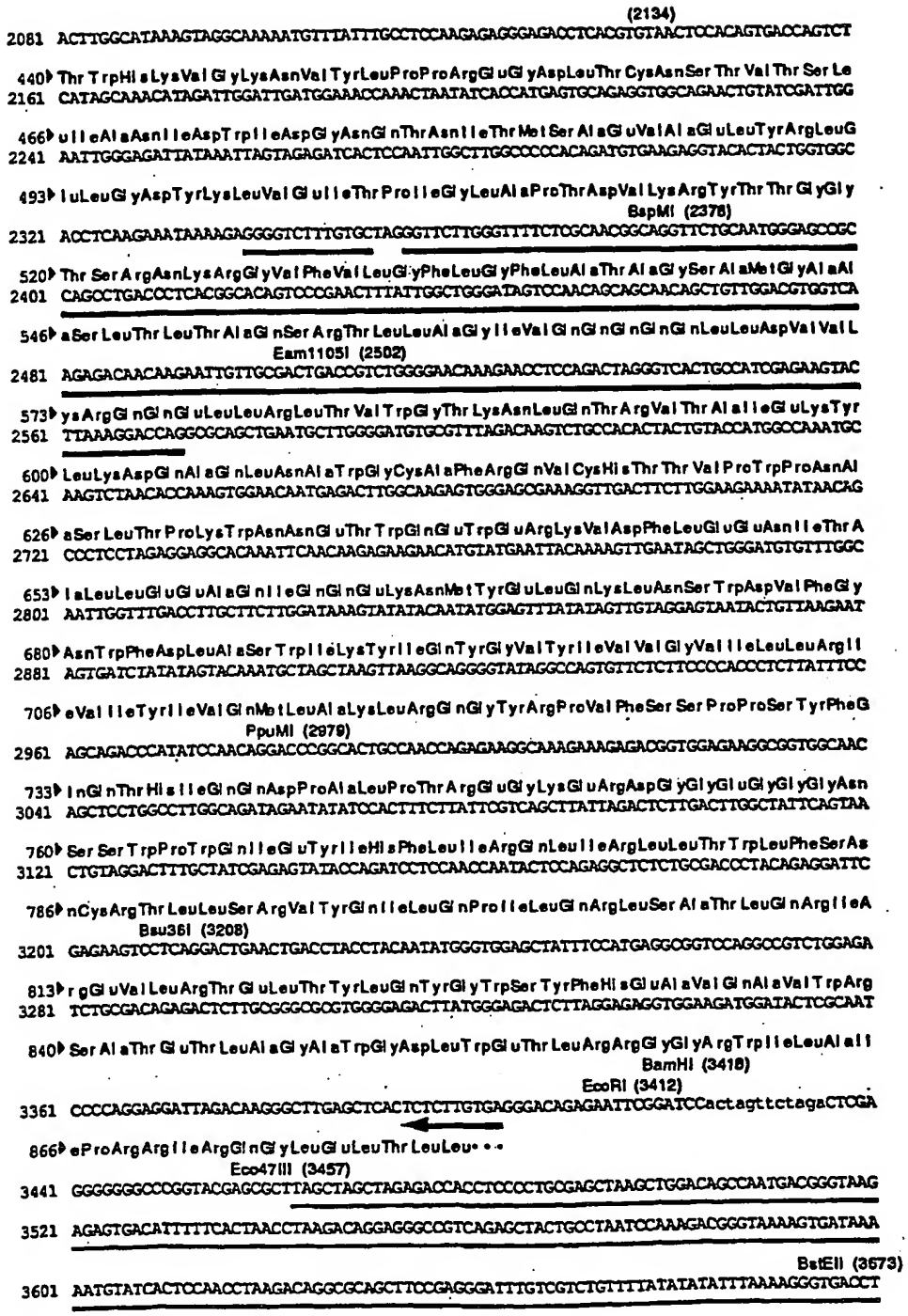

(2134)
2081 ACTTGGCATAAAGTAGGCAAAAATGTTTATTTGCCTCCAAGAGAGGGAGACCTCACGTGTAACTCCACAGTGACCAGTCT
440 Thr TrpHisLysValGlyLysAsnValTyrLeuProProArgGluGlyAspLeuThrCysAsnSerThrValThrSerLe
2161 CATAGCAAACATAGATTGGATTGATGGAAACCAAACTAATATCACCATGAGTGCAGAGGTGGCAGAACTGTATCGATTGG
466 uIleAlaAsnIleAspTrpIleAspGlyAsnGlnThrAsnIleThrMetSerAlaGluValAlaGluLeuTyrArgLeuG
2241 AATTGGGAGATTATAAATTAGTAGAGATCACTCCAATTGGCTTGGCCCCCACAGATGTGAAGAGGTACACTACTGGTGGC
493 luLeuGlyAspTyrLysLeuValGluIleThrProIleGlyLeuAlaProThrAspValLysArgTyrThrThrGlyGly
BspMI (2378)
2321 ACCTCAAGAAATAAAAGAGGGGTCTTTGTGCTAGGGTTCTTGGGTTTTCTGCAACGGCAGGTTCTGCAATGGGAGCCGC
520 Thr SerArgAsnLysArgGlyValPheValLeuGlyPheLeuGlyPheLeuAlaThrAlaGlySerAlaMetGlyAlaAl
2401 CAGCCTGACCCTCACGGCACAGTCCCGAACTTTATTGGCTGGGATAGTCCAACAGCAGCAACAGCTGTTGGACGTGGTCA
546 aSerLeuThrLeuThrAlaGlnSerArgThrLeuLeuAlaGlyIleValGlnGlnGlnGlnLeuLeuAspValValL
Eam1105I (2502)
2481 AGAGACAACAAGAATTGTTGCGACTGACCGTCTGGGGAACAAAGAACCTCCAGACTAGGGTCACTGCCATCGAGAAGTAC
573 ysArgGlnGlnGluLeuLeuArgLeuThrValTrpGlyThrLysAsnLeuGlnThrArgValThrAlaIleGluLysTyr
2561 TTAAAGGACCAGGCGCAGCTGAATGCTTGGGGATGTGCGTTTAGACAAGTCTGCCACACTACTGTACCATGGCCAAATGC
600 LeuLysAspGlnAlaGlnLeuAsnAlaTrpGlyCysAlaPheArgGlnValCysHisThrThrValProTrpProAsnAl
2641 AAGTCTAACACCAAAGTGGAACAATGAGACTTGGCAAGAGTGGGAGCGAAAGGTTGACTTCTTGGAAGAAAATATAACAG
626 aSerLeuThrProLysTrpAsnAsnGluThrTrpGlnGluTrpGluArgLysValAspPheLeuGluGluAsnIleThrA
2721 CCCTCCTAGAGGAGGCACAAATTCAACAAGAGAAGAACATGTATGAATTACAAAAGTTGAATAGCTGGGATGTGTTTGGC
653 laLeuLeuGluGluAlaGlnIleGlnGlnGluLysAsnMetTyrGluLeuGlnLysLeuAsnSerTrpAspValPheGly
2801 AATTGGTTTGACCTTGCTTCTTGGATAAAGTATATACAATATGGAGTTTATATAGTTGTAGGAGTAATACTGTTAAGAAT
680 AsnTrpPheAspLeuAlaSerTrpIleLysTyrIleGlnTyrGlyValTyrIleValValGlyValIleLeuLeuArgIl
2881 AGTGATCTATATAGTACAAATGCTAGCTAAGTTAAGGCAGGGGTATAGGCCAGTGTTCTCTTCCCCACCCTCTTATTTCC
706 eValIleTyrIleValGlnMetLeuAlaLysLeuArgGlnGlyTyrArgProValPheSerSerProProSerTyrPheG
PpuMI (2979)
2961 AGCAGACCCATATCCAACAGGACCCGGCACTGCCAACCAGAGAAGGCAAAGAAAGAGACGGTGGAGAAGGCGGTGGCAAC
733 lnGlnThrHisIleGlnGlnAspProAlaLeuProThrArgGluGlyLysGluArgAspGlyGlyGluGlyGlyGlyAsn
3041 AGCTCCTGGCCTTGGCAGATAGAATATATCCACTTTCTTATTCGTCAGCTTATTAGACTCTTGACTTGGCTATTCAGTAA
760 Ser SerTrpProTrpGlnIleGluTyrIleHisPheLeuIleArgGlnLeuIleArgLeuLeuThrTrpLeuPheSerAs
3121 CTGTAGGACTTTGCTATCGAGAGTATACCAGATCCTCCAACCAATACTCCAGAGGCTCTCTGCGACCCTACAGAGGATTC
786 nCysArgThrLeuLeuSerArgValTyrGlnIleLeuGlnProIleLeuGlnArgLeuSerAlaThrLeuGlnArgIleA
Bsu36I (3208)
3201 GAGAAGTCCTCAGGACTGAACTGACCTACCTACAATATGGGTGGAGCTATTTCCATGAGGCGGTCCAGGCCGTCTGGAGA
813 rgGluValLeuArgThrGluLeuThrTyrLeuGlnTyrGlyTrpSerTyrPheHisGluAlaValGlnAlaValTrpArg
3281 TCTGCGACAGAGACTCTTGCGGGCGCGTGGGGAGACTTATGGGAGACTCTTAGGAGAGGTGGAAGATGGATACTCGCAAT
840 SerAlaThrGluThrLeuAlaGlyAlaTrpGlyAspLeuTrpGluThrLeuArgArgGlyGlyArgTrpIleLeuAlaIl
BamHI (3418)
EcoRI (3412)
3361 CCCCAGGAGGATTAGACAAGGGCTTGAGCTCACTCTCTTGTGAGGGACAGAGAATTCGGATCCactagttctagaCTCGA
866 eProArgArgIleArgGlnGlyLeuGluLeuThrLeuLeu• • •
Eco47III (3457)
3441 GGGGGGGCCCGGTACGAGCGCTTAGCTAGCTAGAGACCACCTCCCCTGCGAGCTAAGCTGGACAGCCAATGACGGGTAAG
3521 AGAGTGACATTTTTCACTAACCTAAGACAGGAGGGCCGTCAGAGCTACTGCCTAATCCAAAGACGGGTAAAAGTGATAAA
BstEII (3673)
3601 AATGTATCACTCCAACCTAAGACAGGCGCAGCTTCCGAGGGATTTGTCGTCTGTTTTATATATATTTAAAAGGGTGACCT

FIGURE 7-2


BsaBI (3740)
SphI (3948)
KpnI (3976)
BstXI (4060)
XmnI (4293)
StuI (5368)
PvuI (5993)
SgfI (5992)
BsrFI (6036)
SspI (6067)
SmaI (6118)
NruI (6395)
DraIII (6523)

FIGURE 7-3

```
6641 TTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAG
6721 GCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTC
6801 TGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTAT
6881 GCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCG
6961 CATCAGATTGGCTATTGG
```

FIGURE 7-4

```
1 ATG AAC CCA AGT GCT GCC GTC ATT TTC TGC CTC ATC CTG CTG GGT CTG AGT GGG ACT CAA
1  M   N   P   S   A   A   V   I   F   C   L   I   L   L   G   L   S   G   T   Q

61 Ggg atc ctc gac ATG GCG CAA CCG GTA GGT ATA AAC ACA AGC ACA ACC TGT TGC TAT CGT
21  G   I   L   D   M   A   Q   P   V   G   I   N   T   S   T   T   C   C   Y   R

121 TTC ATA AAT AAA AAG ATA CCG AAG CAA CGT CTG GAA AGC TAT CGC CGT ACC ACT TCT AGC
41  F   I   N   K   K   I   P   K   Q   R   L   E   S   Y   R   R   T   T   S   S

181 CAC TGT CCG CGT GAA GCT GTT ATA TTC AAA ACG AAA CTG GAT AAG GAG ATC TGC GCC GAC
61  H   C   P   R   E   A   V   I   F   K   T   K   L   D   K   E   I   C   A   D

241 CCT ACA CAG AAA TGG GTT CAG GAC TTT ATG AAG CAC CTG GAT AAA AAG ACA CAG ACG CCG
81  P   T   Q   K   W   V   Q   D   F   M   K   H   L   D   K   K   T   Q   T   P

301 AAA CTG ATC TGC AGC GCC GAG GAG AAG CTG TGG GTC ACG GTC TAT TAT GGC GTG CCC GTG
101 K   L   I   C   S   A   E   E   K   L   W   V   T   V   Y   Y   G   V   P   V

361 TGG AAA GAG GCA ACC ACC ACG CTA TTC TGC GCC TCC GAC GCC AAG GCA CAT CAT GCA GAG
121 W   K   E   A   T   T   T   L   F   C   A   S   D   A   K   A   H   H   A   E

421 GCG CAC AAC GTC TGG GCC ACG CAT GCC TGT GTA CCC ACG GAC CCT AAC CCC CAA GAG GTG
141 A   H   N   V   W   A   T   H   A   C   V   P   T   D   P   N   P   Q   E   V

481 ATC CTG GAG AAC GTG ACC GAG AAG TAC AAC ATG TGG AAA AAT AAC ATG GTA GAC CAG ATG
161 I   L   E   N   V   T   E   K   Y   N   M   W   K   N   N   M   V   D   Q   M

541 CAT GAG GAT ATA ATC AGT CTA TGG GAT CAA AGC CTA AAG CCA TGT GTA AAA CTA ACC CCC
181 H   E   D   I   I   S   L   W   D   Q   S   L   K   P   C   V   K   L   T   P

601 CTC TGC GTG ACG CTG AAT TGC ACC AAC GCG ACG TAT ACG AAT AGT GAC AGT AAG AAT AGT
201 L   C   V   T   L   N   C   T   N   A   T   Y   T   N   S   D   S   K   N   S

661 ACC AGT AAT AGT AGT TTG GAG GAC AGT GGG AAA GGA GAC ATG AAC TGC TCG TTC GAT GTC
221 T   S   N   S   S   L   E   D   S   G   K   G   D   M   N   C   S   F   D   V

721 ACC ACC AGC ATC GAC AAG AAG AAG AAG ACG GAG TAT GCC ATC TTC GAC AAG CTG GAT GTA
241 T   T   S   I   D   K   K   K   K   T   E   Y   A   I   F   D   K   L   D   V

781 ATG AAT ATA GGA AAT GGA AGA TAT ACG CTA TTG AAT TGT AAC ACC AGT GTC ATT ACG CAG
261 M   N   I   G   N   G   R   Y   T   L   L   N   C   N   T   S   V   I   T   Q

841 GCC TGT CCA AAG ATG TCC TTT GAG CCA ATT CCC ATA CAT TAT TGT ACC CCG GCC GGC TAC
281 A   C   P   K   M   S   F   E   P   I   P   I   H   Y   C   T   P   A   G   Y
```

FIGURE 8-1

```
901 GCG ATC CTG AAG TGC AAC GAC AAT AAG TTC AAT GGA ACG GGA CCA TGT ACG AAT GTC AGC
301  A   I   L   K   C   N   D   N   K   F   N   G   T   G   P   C   T   N   V   S

961 ACG ATA CAA TGT ACG CAT GGA ATT AAG CCA GTA GTG TCG ACG CAA CTG CTG CTG AAC GGC
321  T   I   Q   C   T   H   G   I   K   P   V   V   S   T   Q   L   L   L   N   G

1021 AGC CTG GCC GAG GGA GGA GAG GTA ATA ATT CGG TCG GAG AAC CTC ACC GAC AAC GCC AAG
341  S   L   A   E   G   G   E   V   I   I   R   S   E   N   L   T   D   N   A   K

1081 ACC ATA ATA GTA CAG CTC AAG GAA CCC GTG GAG ATC AAC TGT ACG AGA CCC AAC AAC AAC
361  T   I   I   V   Q   L   K   E   P   V   E   I   N   C   T   R   P   N   N   N

1141 ACC CGA AAG AGC ATA CAT ATG GGA CCA GGA GCA GCA TTT TAT GCA AGA GGA GAG GTA ATA
381  T   R   K   S   I   H   M   G   P   G   A   A   F   Y   A   R   G   E   V   I

1201 GGA GAT ATA AGA CAA GCA CAT TGC AAC ATT AGT AGA GGA AGA TGG AAT GAC ACT TTG AAA
401  G   D   I   R   Q   A   H   C   N   I   S   R   G   R   W   N   D   T   L   K

1261 CAG ATA GCT AAA AAG CTG CGC GAG CAG TTT AAC AAG ACC ATA AGC CTT AAC CAA TCC TCG
421  Q   I   A   K   K   L   R   E   Q   F   N   K   T   I   S   L   N   Q   S   S

1321 GGA GGG GAC CTA GAG ATT GTA ATG CAC ACG TTT AAT TGT GGA GGG GAG TTT TTC TAC TGT
441  G   G   D   L   E   I   V   M   H   T   F   N   C   G   G   E   F   F   Y   C

1381 AAC ACG ACC CAG CTG TTC AAC AGC ACC TGG AAT GAG AAT GAT ACG ACC TGG AAT AAT ACG
461  N   T   T   Q   L   F   N   S   T   W   N   E   N   D   T   T   W   N   N   T

1441 GCA GGG TCG AAT AAC AAT GAG ACG ATC ACC CTG CCC TGT CGC ATC AAG CAG ATC ATA AAC
481  A   G   S   N   N   N   E   T   I   T   L   P   C   R   I   K   Q   I   I   N

1501 AGG TGG CAG GAA GTA GGA AAA GCA ATG TAT GCC CCT CCC ATC AGT GGC CCG ATC AAC TGC
501  R   W   Q   E   V   G   K   A   M   Y   A   P   P   I   S   G   P   I   N   C

1561 TTG TCC AAC ATC ACC GGG CTA TTG TTG ACG AGA GAT GGT GGT GAC AAC AAT AAT ACG ATA
521  L   S   N   I   T   G   L   L   L   T   R   D   G   G   D   N   N   N   T   I

1621 GAG ACC TTC AGA CCT GGA GGA GGA GAT ATG AGG GAC AAC TGG AGG AGC GAG CTG TAC AAG
541  E   T   F   R   P   G   G   G   D   M   R   D   N   W   R   S   E   L   Y   K
```

FIGURE 8-2

```
1681 TAC AAG GTA GTG AGG ATC GAG CCA TTG GGA ATA GCA CCC ACC AAG GCA AAG AGA AGA GTG
561▶ Y   K   V   V   R   I   E   P   L   G   I   A   P   T   K   A   K   R   R   V

1741 GTG CAA AGA GAG AAA AGA GCA GTG GGA ATA GGA GCT ATG TTC CTT GGG TTC TTG GGA GCA
581▶ V   Q   R   E   K   R   A   V   G   I   G   A   M   F   L   G   F   L   G   A

1801 GCA GGA AGC ACT ATG GGC GCA GCG TCG GTG ACC CTT ACC GTG CAA GCT CGC CTG CTG CTG
601▶ A   G   S   T   M   G   A   A   S   V   T   L   T   V   Q   A   R   L   L   L

1861 TCG GGT ATA GTG CAA CAG CAA AAC AAC CTC CTC CGC GCA ATC GAA GCC CAG CAG CAT CTG
621▶ S   G   I   V   Q   Q   Q   N   N   L   L   R   A   I   E   A   Q   Q   H   L

1921 TTG CAA CTC ACG GTC TGG GGC ATC AAG CAG CTC CAG GCT AGA GTC CTT GCC ATG GAG CGT
641▶ L   Q   L   T   V   W   G   I   K   Q   L   Q   A   R   V   L   A   M   E   R

1981 TAT CTG AAA GAC CAG CAA CTT CTT GGG ATT TGG GGT TGC TCG GGA AAA CTC ATT TGC ACC
661▶ Y   L   K   D   Q   Q   L   L   G   I   W   G   C   S   G   K   L   I   C   T

2041 ACG AAT GTG CCT TGG AAC GCC AGC TGG AGC AAC AAG TCC CTG GAC AAG ATT TGG CAT AAC
681▶ T   N   V   P   W   N   A   S   W   S   N   K   S   L   D   K   I   W   H   N

2101 ATG ACC TGG ATG GAG TGG GAC CGC GAG ATC GAC AAC TAC ACG AAA TTG ATA TAC ACC CTG
701▶ M   T   W   M   E   W   -D  R   E   I   D   N   Y   T   K   L   I   Y   T   L

2161 ATC GAG GCG TCC CAG ATC CAG CAG GAG AAG AAT GAG CAA GAG TTG TTG GAG TTG GAT TCG
721▶ I   E   A   S   Q   I   Q   Q   E   K   N   E   Q   E   L   L   E   L   D   S

2221 TGG GCG TCG TTG TGG TCG TGG TTT GAC ATC TCG AAA TGG CTG TGG TAT ATA GGA GTA TTC
741▶ W   A   S   L   W   S   W   F   D   I   S   K   W   L   W   Y   I   G   V   F

2281 ATA ATA GTA ATA GGA GGT TTG GTA GGT TTG AAA ATA GTT TTT GCT GTA CTT TCG ATA GTA
761▶ I   I   V   I   G   G   L   V   G   L   K   I   V   F   A   V   L   S   I   V

2341 AAT CGA GTT AGG CAG GGA TAC TCG CCA TTG TCA TTT CAA ACC CGC CTC CCA GCC CCG CGG
781▶ N   R   V   R   Q   G   Y   S   P   L   S   F   Q   T   R   L   P   A   P   R

2401 GGA CCC GAC AGG CCC GAG GGC ATC GAG GAG GGA GGC GGC GAG AGA GAC AGA GAC AGA TCC
801▶ G   P   D   R   P   E   G   I   E   E   G   G   G   E   R   D   R   D   R   S
```

FIGURE 8-3

```
2461 GAT CAA TTG GTG ACG GGA TTC TTG GCA CTC ATC TGG GAC GAT CTG CGG AGC CTG TGC CTC
 821▶ D  Q  L  V  T  G  F  L  A  L  I  W  D  D  L  R  S  L  C  L

2521 TTC TCT TAC CAC CGC CTG CGC GAC CTG CTC CTG ATC GTG GCG AGG ATC GTG GAG CTT CTG
 841▶ F  S  Y  H  R  L  R  D  L  L  L  I  V  A  R  I  V  E  L  L

2581 GGA CGC AGG GGG TGG GAG GCC CTG AAG TAC TGG TGG AAC CTC CTG CAA TAT TGG ATT CAG
 861▶ G  R  R  G  W  E  A  L  K  Y  W  W  N  L  L  Q  Y  W  I  Q

2641 GAG CTG AAG AAC AGC GCC GTT AGT CTG CTG AAC GCT ACC GCT ATC GCC GTG GCG GAA GGA
 881▶ E  L  K  N  S  A  V  S  L  L  N  A  T  A  I  A  V  A  E  G

2701 ACC GAC AGG ATT ATA GAG GTA GTA CAA AGG ATT GGT CGC GCC ATC CTC CAT ATC CCC CGC
 901▶ T  D  R  I  I  E  V  V  Q  R  I  G  R  A  I  L  H  I  P  R

2761 CGC ATC CGC CAG GGC TTG GAG AGG GCT TTG CTA TAA
 921▶ R  I  R  Q  G  L  E  R  A  L  L  •
```

FIGURE 8-4

```
   1 ATG AGA AAA GCG GCT GTT AGT CAC TGG CAG CAG CAG TCT TAC CTG GAC TCT GGA ATC CAT
   1▶ M   R   K   A   A   V   S   H   W   Q   Q   Q   S   Y   L   D   S   G   I   H
   1▶ M   R   K   A   A   V   S   H   W   Q   Q   Q   S   Y   L   D   S   G   I   H

  61 TCT GGT GCC ACT ACC ACA GCT CCT TCT CTG AGT ATC TGC AGC GCC GAG GAG AAG CTG TGG
  21▶ S   G   A   T   T   T   A   P   S   L   S
  21▶ S   G   A   T   T   T   A   P   S   L   S   I   C   S   A   E   E   K   L   W

 121 GTC ACG GTC TAT TAT GGC GTG CCC GTG TGG AAA GAG GCA ACC ACC ACG CTA TTC TGC GCC

  41▶ V   T   V   Y   Y   G   V   P   V   W   K   E   A   T   T   T   L   F   C   A

 181 TCC GAC GCC AAG GCA CAT CAT GCA GAG GCG CAC AAC GTC TGG GCC ACG CAT GCC TGT GTA

  61▶ S   D   A   K   A   H   H   A   E   A   H   N   V   W   A   T   H   A   C   V

 241 CCC ACG GAC CCT AAC CCC CAA GAG GTG ATC CTG GAG AAC GTG ACC GAG AAG TAC AAC ATG

  81▶ P   T   D   P   N   P   Q   E   V   I   L   E   N   V   T   E   K   Y   N   M

 301 TGG AAA AAT AAC ATG GTA GAC CAG ATG CAT GAG GAT ATA ATC AGT CTA TGG GAT CAA AGC

 101▶ W   K   N   N   M   V   D   Q   M   H   E   D   I   I   S   L   W   D   Q   S

 361 CTA AAG CCA TGT GTA AAA CTA ACC CCC CTC TGC GTG ACG CTG AAT TGC ACC AAC GCG ACG

 121▶ L   K   P   C   V   K   L   T   P   L   C   V   T   L   N   C   T   N   A   T

 421 TAT ACG AAT AGT GAC AGT AAG AAT AGT ACC AGT AAT AGT AGT TTG GAG GAC AGT GGG AAA

 141▶ Y   T   N   S   D   S   K   N   S   T   S   N   S   S   L   E   D   S   G   K

 481 GGA GAC ATG AAC TGC TCG TTC GAT GTC ACC ACC AGC ATC GAC AAG AAG AAG AAG ACG GAG

 161▶ G   D   M   N   C   S   F   D   V   T   T   S   I   D   K   K   K   K   T   E

 541 TAT GCC ATC TTC GAC AAG CTG GAT GTA ATG AAT ATA GGA AAT GGA AGA TAT ACG CTA TTG

 181▶ Y   A   I   F   D   K   L   D   V   M   N   I   G   N   G   R   Y   T   L   L

 601 AAT TGT AAC ACC AGT GTC ATT ACG CAG GCC TGT CCA AAG ATG TCC TTT GAG CCA ATT CCC

 201▶ N   C   N   T   S   V   I   T   Q   A   C   P   K   M   S   F   E   P   I   P

 661 ATA CAT TAT TGT ACC CCG GCC GGC TAC GCG ATC CTG AAG TGC AAC GAC AAT AAG TTC AAT

 221▶ I   H   Y   C   T   P   A   G   Y   A   I   L   K   C   N   D   N   K   F   N

 721 GGA ACG GGA CCA TGT ACG AAT GTC AGC ACG ATA CAA TGT ACG CAT GGA ATT AAG CCA GTA

 241▶ G   T   G   P   C   T   N   V   S   T   I   Q   C   T   H   G   I   K   P   V

 781 GTG TCG ACG CAA CTG CTG CTG AAC GGC AGC CTG GCC GAG GGA GGA GAG GTA ATA ATT CGG

 261▶ V   S   T   Q   L   L   L   N   G   S   L   A   E   G   G   E   V   I   I   R

 841 TCG GAG AAC CTC ACC GAC AAC GCC AAG ACC ATA ATA GTA CAG CTC AAG GAA CCC GTG GAG

 281▶ S   E   N   L   T   D   N   A   K   T   I   I   V   Q   L   K   E   P   V   E

 901 ATC AAC TGT ACG AGA CCC AAC AAC AAC ACC CGA AAG AGC ATA CAT ATG GGA CCA GGA GCA

 301▶ I   N   C   T   R   P   N   N   N   T   R   K   S   I   H   M   G   P   G   A

 961 GCA TTT TAT GCA AGA GGA GAG GTA ATA GGA GAT ATA AGA CAA GCA CAT TGC AAC ATT AGT

 321▶ A   F   Y   A   R   G   E   V   I   G   D   I   R   Q   A   H   C   N   I   S
```

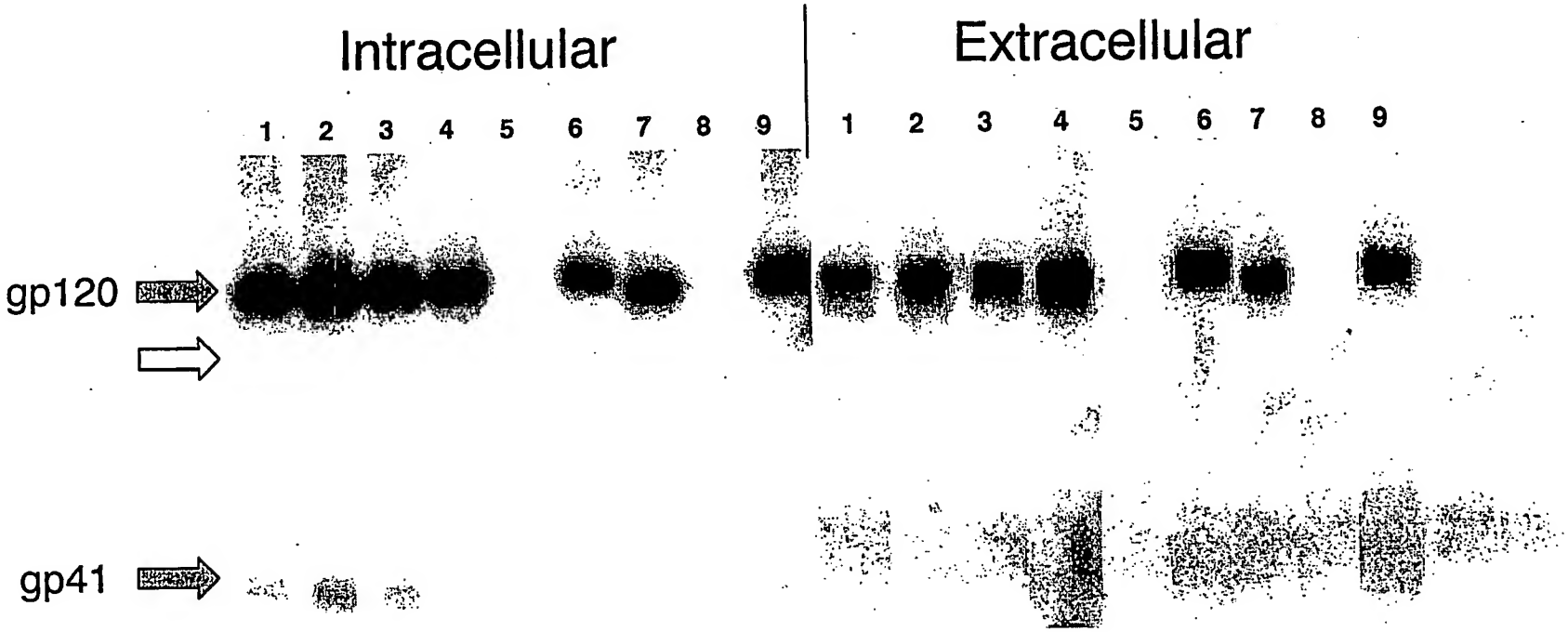
FIGURE 9-1

```
1021 AGA GGA AGA TGG AAT GAC ACT TTG AAA CAG ATA GCT AAA AAG CTG CGC GAG CAG TTT AAC
 341▶ R   G   R   W   N   D   T   L   K   Q   I   A   K   K   L   R   E   Q   F   N
1081 AAG ACC ATA AGC CTT AAC CAA TCC TCG GGA GGG GAC CTA GAG ATT GTA ATG CAC ACG TTT
 361▶ K   T   I   S   L   N   Q   S   S   G   G   D   L   E   I   V   M   H   T   F
1141 AAT TGT GGA GGG GAG TTT TTC TAC TGT AAC ACG ACC CAG CTG TTC AAC AGC ACC TGG AAT
 381▶ N   C   G   G   E   F   F   Y   C   N   T   T   Q   L   F   N   S   T   W   N
1201 GAG AAT GAT ACG ACC TGG AAT AAT ACG GCA GGG TCG AAT AAC AAT GAG ACG ATC ACC CTG
 401▶ E   N   D   T   T   W   N   N   T   A   G   S   N   N   N   E   T   I   T   L
1261 CCC TGT CGC ATC AAG CAG ATC ATA AAC AGG TGG CAG GAA GTA GGA AAA GCA ATG TAT GCC
 421▶ P   C   R   I   K   Q   I   I   N   R   W   Q   E   V   G   K   A   M   Y   A
1321 CCT CCC ATC AGT GGC CCG ATC AAC TGC TTG TCC AAC ATC ACC GGG CTA TTG TTG ACG AGA
 441▶ P   P   I   S   G   P   I   N   C   L   S   N   I   T   G   L   L   L   T   R
1381 GAT GGT GGT GAC AAC AAT AAT ACG ATA GAG ACC TTC AGA CCT GGA GGA GGA GAT ATG AGG
 461▶ D   G   G   D   N   N   N   T   I   E   T   F   R   P   G   G   G   D   M   R
1441 GAC AAC TGG AGG AGC GAG CTG TAC AAG TAC AAG GTA GTG AGG ATC GAG CCA TTG GGA ATA
 481▶ D   N   W   R   S   E   L   Y   K   Y   K   V   V   R   I   E   P   L   G   I
1501 GCA CCC ACC AAG GCA AAG AGA AGA GTG GTG CAA AGA GAG AAA AGA GCA GTG GGA ATA GGA
 501▶ A   P   T   K   A   K   R   R   V   V   Q   R   E   K   R   A   V   G   I   G
1561 GCT ATG TTC CTT GGG TTC TTG GGA GCA GCA GGA AGC ACT ATG GGC GCA GCG TCG GTG ACC
 521▶ A   M   F   L   G   F   L   G   A   A   G   S   T   M   G   A   A   S   V   T
1621 CTT ACC GTG CAA GCT CGC CTG CTG CTG TCG GGT ATA GTG CAA CAG CAA AAC AAC CTC CTC
 541▶ L   T   V   Q   A   R   L   L   L   S   G   I   V   Q   Q   Q   N   N   L   L
1681 CGC GCA ATC GAA GCC CAG CAG CAT CTG TTG CAA CTC ACG GTC TGG GGC ATC AAG CAG CTC
 561▶ R   A   I   E   A   Q   Q   H   L   L   Q   L   T   V   W   G   I   K   Q   L
1741 CAG GCT AGA GTC CTT GCC ATG GAG CGT TAT CTG AAA GAC CAG CAA CTT CTT GGG ATT TGG
 581▶ Q   A   R   V   L   A   M   E   R   Y   L   K   D   Q   Q   L   L   G   I   W
1801 GGT TGC TCG GGA AAA CTC ATT TGC ACC ACG AAT GTG CCT TGG AAC GCC AGC TGG AGC AAC
 601▶ G   C   S   G   K   L   I   C   T   T   N   V   P   W   N   A   S   W   S   N
1861 AAG TCC CTG GAC AAG ATT TGG CAT AAC ATG ACC TGG ATG GAG TGG GAC CGC GAG ATC GAC
 621▶ K   S   L   D   K   I   W   H   N   M   T   W   M   E   W   D   R   E   I   D
1921 AAC TAC ACG AAA TTG ATA TAC ACC CTG ATC GAG GCG TCC CAG ATC CAG CAG GAG AAG AAT
 641▶ N   Y   T   K   L   I   Y   T   L   I   E   A   S   Q   I   Q   Q   E   K   N
1981 GAG CAA GAG TTG TTG GAG TTG GAT TCG TGG GCG TCG TTG TGG TCG TGG TTT GAC ATC TCG
 661▶ E   Q   E   L   L   E   L   D   S   W   A   S   L   W   S   W   F   D   I   S
```

FIGURE 9-2

```
2041 AAA TGG CTG TGG TAT ATA GGA GTA TTC ATA ATA GTA ATA GGA GGT TTG GTA GGT TTG AAA
 681▶ K   W   L   W   Y   I   G   V   F   I   I   V   I   G   G   L   V   G   L   K
2101 ATA GTT TTT GCT GTA CTT TCG ATA GTA AAT CGA GTT AGG CAG GGA TAC TCG CCA TTG TCA
 701▶ I   V   F   A   V   L   S   I   V   N   R   V   R   Q   G   Y   S   P   L   S
2161 TTT CAA ACC CGC CTC CCA GCC CCG CGG GGA CCC GAC AGG CCC GAG GGC ATC GAG GAG GGA
 721▶ F   Q   T   R   L   P   A   P   R   G   P   D   R   P   E   G   I   E   E   G
2221 GGC GGC GAG AGA GAC AGA GAC AGA TCC GAT CAA TTG GTG ACG GGA TTC TTG GCA CTC ATC
 741▶ G   G   E   R   D   R   D   R   S   D   Q   L   V   T   G   F   L   A   L   I
2281 TGG GAC GAT CTG CGG AGC CTG TGC CTC TTC TCT TAC CAC CGC CTG CGC GAC CTG CTC CTG
 761▶ W   D   D   L   R   S   L   C   L   F   S   Y   H   R   L   R   D   L   L   L
2341 ATC GTG GCG AGG ATC GTG GAG CTT CTG GGA CGC AGG GGG TGG GAG GCC CTG AAG TAC TGG
 781▶ I   V   A   R   I   V   E   L   L   G   R   R   G   W   E   A   L   K   Y   W
2401 TGG AAC CTC CTG CAA TAT TGG ATT CAG GAG CTG AAG AAC AGC GCC GTT AGT CTG CTG AAC
 801▶ W   N   L   L   Q   Y   W   I   Q   E   L   K   N   S   A   V   S   L   L   N
2461 GCT ACC GCT ATC GCC GTG GCG GAA GGA ACC GAC AGG ATT ATA GAG GTA GTA CAA AGG ATT
 821▶ A   T   A   I   A   V   A   E   G   T   D   R   I   I   E   V   V   Q   R   I
2521 GGT CGC GCC ATC CTC CAT ATC CCC CGC CGC ATC CGC CAG GGC TTG GAG AGG GCT TTG CTA
 841▶ G   R   A   I   L   H   I   P   R   R   I   R   Q   G   L   E   R   A   L   L
2581 TAA
```

FIGURE 9-3



1 - 6101 mod.
2 - 6101 mod.CTE
3 - 6101 mod. RTE
4 - tPA-6101 mod.CTE
5 - cate-6101 mod.
6 - MCP3-6101mod.CTE
7 - tPA-6101 mod.RTE
8 - cate-6101 mod.RTE
9 - MCP3-6101mod.RTE

FIGURE 10